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EFFECTS OF ANTS ON RIPARIAN POPLARS: AN EX SITU EXPERIMENT OF BIOTIC INTERACTION

D. Corenblit^{1,3*}, B. Corbara², R. Cereghino³, A. Dejean³, P. Duran¹, V. Garófano-Gómez^{1,4}, F. Gilbert³, E. González-Sargas⁵, F. Julien³, L. Lambs³, C. Mainguin³, L. Mazal¹, T. Otto³, J. Steiger¹, E. Tabacchi³, I. Till-Bottraud¹, Y. Travaillard³

¹ Université Clermont Auvergne, CNRS, GEOLAB, F-63000 Clermont-Ferrand, France

² Université Clermont Auvergne, CNRS, LMGE, F-63000 Clermont-Ferrand, France

³ Laboratoire Écologie Fonctionnelle et Environnement, Université de Toulouse, CNRS, Toulouse INP, Université Toulouse 3 – Paul Sabatier (UPS), Toulouse, France

⁴ Institut d'Investigació per a la Gestió Integrada de Zones Costaneres (IGIC), Universitat Politècnica de València, Paranímf 1, Grau de Gandia, 46730 Valencia, Spain

⁵ Department of Biology, Colorado State University; Cooperator with the U.S. Geological Survey Fort Collins Science Center, Fort Collins, CO 80526, USA

*Correspondence to: dov.corenblit@uca.fr

Abstract

Poplars establish on alluvial bars within sand and gravel-bed rivers. Alluvial bars also provide particularly suitable habitats for the proliferation of ants. We hypothesized that ants, by modifying substrate structure and resource availability in fluvial habitats, positively influence poplar growth during its establishment stage. We conducted a preliminary nine-month ex situ greenhouse experiment with one ant species (*Lasius niger* L.) and six different genotypes of poplar cuttings (*Populus nigra* L.), both collected on the Garonne River, SW France. Three main treatments: 'P. nigra alone', 'P. nigra without ants and with ant food' and 'P. nigra with ants and ant food' were applied. After one growing season, we tested differences in branching length and biomass of stems, roots and leaves. Certain genotypes showed significant differences in growth, but there were no significant differences in stem length, dry mass of stems and roots between the three treatments. The total biomass of poplars after the first growing season was positively affected by the initial size of the cuttings and was modulated by the genotype independently from the treatments. However, an increased poplar growth for the treatment without ants and with ant food was observed according to significant differences in dry weight of leaves and total biomass (i.e. dry mass of stems, roots and leaves) for the pooled genotypes across treatments. We discuss our results with the aim of serving as a reference for future in situ and ex situ experiments and field measurements exploring interactions between ants and poplars, specifically in riparian ecosystems.

Keywords

Ex situ experiment, *Populus nigra*, Cuttings, Ant, Plant–animal interaction, *Lasius niger*

1 Introduction

Among typical pioneer trees, poplars are foundation species in riparian forests of temperate biomes ([Isebrands and Richardson 2014](#)), and among poplars, *Populus nigra* L., the black poplar, is the most widespread and dominant pioneer tree growing in European rivers ([Lefèvre et al. 1998](#)). Together with other members of the *Salicaceae* family, they are adapted to physical disturbances caused by floods and sediment instability. They establish on bare alluvial bars, flood deposits, and in secondary channels that recurrently form within dynamic sand and gravel river beds during high-flow events ([Bradley and Smith 1986](#); [Scott et al. 1996](#); [Karrenberg et al. 2002](#); [Francis et al. 2005](#); [Rood et al. 2007](#); [Stella et al. 2011](#)). During the recruitment stage, *P. nigra* seedlings and saplings are subjected to high biomechanical and physiological pressures related to shear stress, erosion and drowning during floods, deposition of sediments and burial, low nutrient availability, and low soil moisture during summer droughts. Because of these constraints, very few among the billions of seeds that germinate on alluvial bars every spring successfully survive the first year and establish in the long term ([Johnson 2000](#); [Karrenberg et al. 2002](#)).

Poplars have been widely studied to understand their responses to mechanical and physiological stress ([González et al. 2012](#); [Singer et al. 2013](#); [Viger et al. 2016](#); [Ludovisi et al. 2017](#)), their biophysical effects on the environment ([Gurnell et al. 2012](#); [Corenblit et al. 2014](#); [Gurnell 2014](#); [Wintenberger et al. 2015](#); [Corenblit et al. 2016a](#); [Diehl et al. 2017](#)), as well as their population genetics and reproductive ecology ([Chenault et al. 2011](#); [González et al. 2016](#); [Bourgeois and González 2019](#); [Mazal et al. 2021](#)). Several studies also focussed on the positive (e.g. facilitation, cooperation, symbiosis) or negative (e.g. grazing, parasitism) interactions between poplars and other taxa, including bacteria ([Doty et al. 2005](#)), mycorrhizae ([Gryta et al. 2006](#); [Newcombe et al. 2010](#)), parasitic Hymenoptera ([Clavijo McCormick et al. 2014](#)), aphids ([Body et al. 2019](#)), mammalian grazers ([Säumel et al. 2011](#)) and beavers ([Laub et al. 2020](#)). However, although some ant species and poplars share the same habitat, i.e. fluvial sand and gravel bars, relationships between poplar ecology and ants have been overlooked.

Abundant in most terrestrial ecosystems, ants are major ecosystem engineers playing a geomorphological role via the construction of mounds and galleries in the soil ([Del Toro et al. 2012](#); [De Almeida et al. 2020](#); [De Souza and Delabie 2020](#); [Viles et al. 2021](#)). They affect soil physicochemical properties by increasing carbon and nutrient (N, P, K) contents through the relocation and accumulation of organic matter and alkalization. They also stimulate microbial and mycorrhizal communities' activity, organic matter decomposition rates and can, directly and indirectly, provide nutrients to plants ([Dauber et al. 2008](#); [Frouz and Jilková 2008](#); [Gonçalves et al. 2016](#); [Bétard 2021](#)). They have been used as indicators of soil quality and anthropogenic impacts on riparian forests ([Segat et al. 2017](#); [Jiménez-Carmona et al. 2020](#)). In addition to these examples of ant effects on trees, ants regulate honeydew-producing hemipterans ([Fischer and Shingleton 2001](#); [Offenberg 2001](#)) and defoliating insects ([Rico-Gray and Oliveira 2008](#)), suggesting that they could positively influence poplar establishment and growth.

Riparian ecosystems provide particularly suitable landforms for the proliferation of certain ant species ([Tagwireyi and Sullivan 2016](#); [Zina et al. 2021](#)), as they can establish in riparian ecosystems and complete the whole or at least a part of their life cycles on alluvial bars exposed to floods. They generally form dense colonies whose workers dig their nests into the sediment and forage on the bars ([Milford 1999](#); [Ballinger et al. 2007](#); [Mertl et al. 2009](#); [Tagwireyi and Sullivan 2016](#)). Some species, such as *Lasius niger* L., can survive the flooding of their habitat ([Hertzog et al. 2016](#)). The nest density of some *Formica* species regularly attains 30 nests per 100 m² ([Lude et al. 1999](#)), a value exceeded by the highly riparian specialized *Cardiocondyla elegans* L., with 100 nests per 100 m² ([Lenoir et al. 2006](#)).

Given that ants and poplars thrive in the same fluvial habitat in high density, we hypothesized that the activity of ants, i.e. the modification of the substrate structure and the availability of resources for plant growth, may influence the survival and growth of poplars during its establishment stage. Based on the literature review, we assumed that the influence could be positive (facilitation) at the seedling stage. Thus, this study aimed to test through an ex situ experimentation the effect of the presence of ants on the growth

of poplar plants, in the form of cuttings originated from different genotypes and during their first growing season.

2 Material and methods

2.1 Study site

Poplar cuttings and ants were collected in March and May 2017, respectively, on a point bar of the Upper Garonne River, France, 20 km downstream of the city of Toulouse. This point bar (ca. 700 m long and 100 m wide at its maximum; 43° 47' 23" N; 1° 17' 16" E; 490 m a.s.l.), constituted of silt, sand, gravel and pebbles, was colonized by pioneer herbaceous and young woody plants dominated by *P. nigra* ([Tabacchi et al. 2019](#)).

2.2 Poplar cuttings

Twigs from young branches of six trees situated more than 20 m apart were collected on the point bar. We also collected buds from each tree and stored them in small plastic flasks with silica gel for later DNA extraction to ensure that we did not collect clones among the six trees. Eight unlinked codominant microsatellites (SSR) markers were used to genotype the trees: WPMS13; WPMS22; PMGC14; PMGC93; PMGC2385; PMGC2578; ORMP221; GCPM2995, for the detailed genetic procedure, see Mazal et al. ([2021](#)). The genetic analysis confirmed that the six trees were distinct genets (hereafter A, B, C, D, E and F).

In the laboratory, the poplar twigs were cut into 12-cm-long cuttings, weighted and their diameters were measured at their two ends and their centre. The mean diameter and weight were used as covariates in the statistical analyses.

2.3 Ants

The most abundant ant species we observed on the study site was black garden ant, also known as common black ant (*Lasius niger*, *Formicinae*), whose colonies, monogynous (each colony has one queen) with monomorphic workers, are abundant in various types of open and sunny environments. They are able to process from 26 to 50 m³/ha of soil with a total volume of anthills reaching 270 m³/ha in alluvial deposits, meadows or abandoned croplands ([Zryanin 2003](#); [Golichenkov et al. 2009](#); [Okrutniak et al. 2020](#)). *L. niger* improves substrate porosity, regulates soil temperature and moisture, causes changes towards neutral pH values and increases nutrient content, mainly N and P. The chemical effects are related to the accumulation of food in the nest and the resulting positive effects on decomposition rates and regulation of microbial activity ([Frouz et al. 2003](#); [Holec and Frouz 2006](#)). Furthermore, colonies can resist continuous flooding for up to three weeks ([Arndt et al. 2011](#); [Hertzog et al. 2016](#)).

In May 2017, using shovels, we collected one *L. niger* colony with its sandy/silty substrate on the aforementioned point bar, placed it in ten large pots of 10 L, each containing thousands of workers and brood, and transported them to the greenhouse. To prevent ants from escaping, these pots were placed in rectangular plastic buckets permanently filled with 10 cm of water and with inner bucket walls regularly brushed with paraffin oil. Plastic bridges between pots allowed ants to travel between them. These ten pots served as a reserve of ants and brood all along the experiment. The permanent presence of incompletely pigmented young workers and abundant brood indicated that the queen was active.

Ants were provided with synthetic food adapted from Dussutour and Simpson ([2012](#)) twice a week. It consisted of agar-agar (8 g), egg (2 g), protein mixture (36 g), sugar (75 g), honey (100 g), vitamins (2 g), salt (2 pinches), inositol (0.50 g), choline chloride (0.50 g) and ascorbic acid (1 g).

2.4 Experimental design

An ex situ experiment was conducted in a thermoregulated greenhouse between March and November 2017. It was designed to test the effect of ant presence on the growth of poplar cuttings originating from six genotypes and growing in semi-controlled conditions over several months (Fig. 1a). One cutting per genotype for a total of six cuttings were randomly planted in a circular pot filled with exactly 28 kg of sand collected from the floodplain of the Garonne River, sieved to a texture of less than 4 mm and well mixed for homogeneity (Fig. 1b). The initial chemical properties of the sediment were determined (Table 1). Resistant polymeric microgrids (mesh size = 0.25 mm) were disposed at the bottom of the pots in order to prevent ants from escaping through the drainage holes. An automatic irrigation system was placed above the pots and programmed with a 1-min-long aerial aspersion at 6 a.m. every 2 days. Based on the test of the volume of water that each pot was receiving from the irrigation system, this corresponded to the minimum duration required to complete full imbibition of the sediments of all the pots.

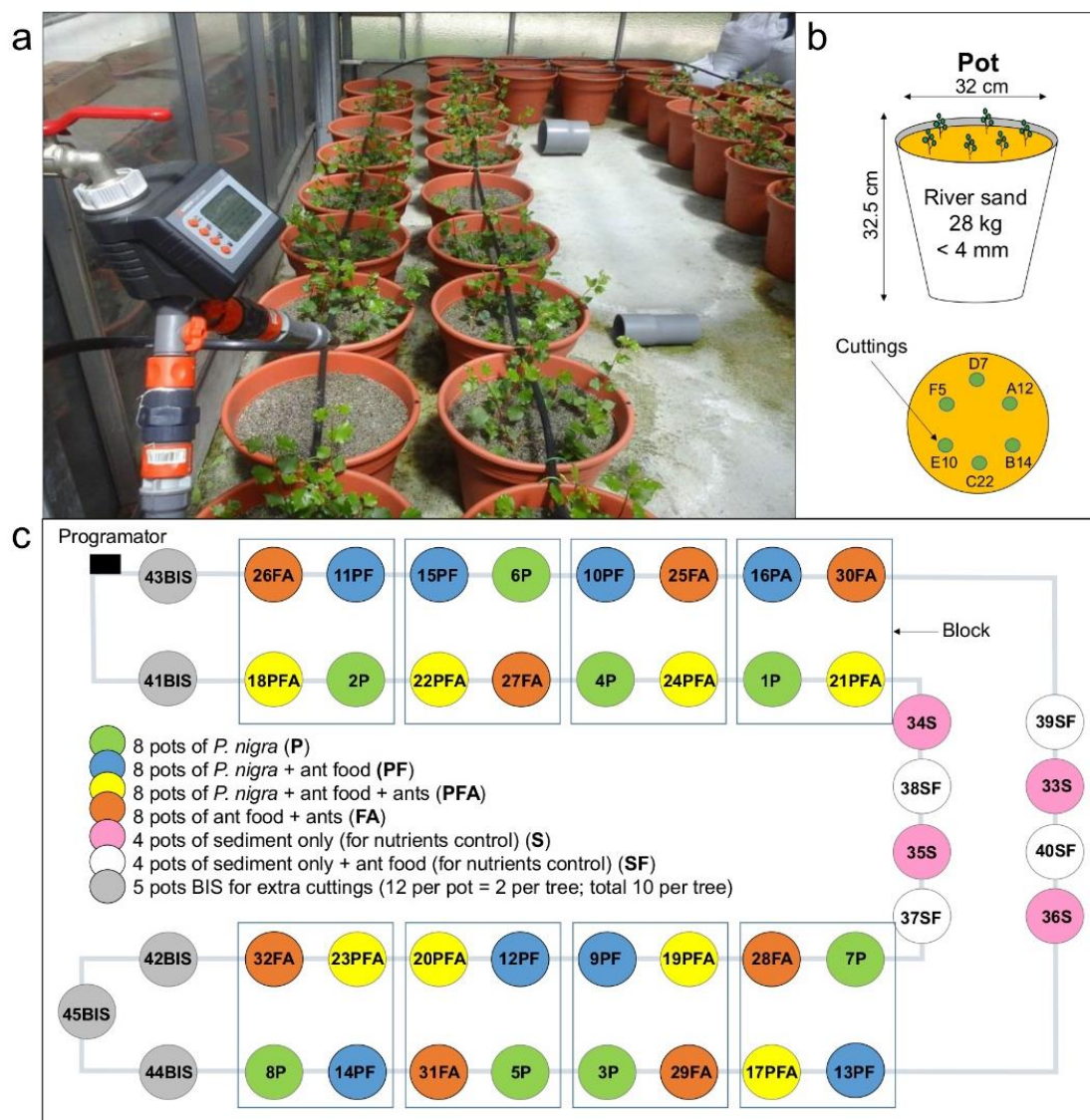


Fig. 1 **a** Photo of the greenhouse with the experimental pots; the grey PVC tubes on the floor contained two data loggers (Gemini™ Tinytags) that monitored temperature and humidity during the entire experiment; **b** Pot dimensions and example of the arrangement of cuttings within each pot; **c** Experimental layout in the greenhouse. Each pot is characterized by a number, a code and a colour. Numbers are unique identifiers of the 40 pots, ordered by treatment. Codes correspond to the six treatments (see Material and methods section and legend). The grey pots 'BIS' correspond

to five extra pots with 12 extra cuttings in each pot (10 extra cuttings per each of the six trees) that were kept as a backup; the grey line represents the irrigation system; blue rectangles represent the eight blocks.

Six treatments were applied to 40 pots (**Fig. 1c**): (1) eight pots '*P. nigra* alone' (P); (2) eight pots '*P. nigra* without ants and with ant food' (PF); (3) eight pots '*P. nigra* with ants and ant food' (PFA); (4) eight pots '*without P. nigra* but with ants and ant food' (FA); (5) four control pots '*with sand only*' (S); and (6) four additional control pots '*with ant food only*' (SF). In each pot with *P. nigra*, six cuttings were planted, one per genotype (**Fig. 1b**). The 32 pots for the four treatments with ants and/or poplars (P, PF, PFA and FA) were randomly distributed and nested within eight blocks to randomize unlikely effects of location within the greenhouse (e.g. orientation, light exposure) on poplar growth. The nutrient controls with no ants and no poplars (S and SF) were placed at the extremity of the greenhouse for logistical reasons; spatial randomization was deemed unnecessary as nutrient levels in the pots did not depend on the greenhouse location. The three main treatments with poplars (P, PF and PFA) were then used for testing differences in poplar growth. The other treatments (FA, S and SF) were used as controls for nutrient content. Additionally, five pots were kept with extra cuttings at the beginning and the end of the irrigation system (BIS) as a backup.

Air temperature and humidity were monitored continuously using two Tinytags (Gemini™, TGP-4500) placed in PVC tubes to avoid direct sunlight: mean temperature (°C) of 20.66 ± 4.71 SD (max: 40.6, min: 6.7); mean air relative humidity of $82.4 \pm 12.5\%$ (max: 100, min: 25.6). The light conditions in the greenhouse were kept homogeneous using daylight fluorescent tubes.

The length of the branches was measured between May and November 2017 (for 13 weeks, 12 bi-monthly series of measures). As cuttings branched during the experiment, the sum of the length of the stems (and all branches) of each plant was noted to determine their growth over the course of the experiment. At the end of the experiment in late November, after one growing season, the entire plants (i.e. stems, roots and leaves) were extracted from the pots using a trickle of water to preserve the root systems and were washed on a metallic grid. The aerial part (stems and branches) was then separated from the roots and the roots were further washed to remove the sediment that remained agglomerated using an ultrasonic cleaning device (Technett, T30 SBF multi-frequency 25/45 kHz) combined with a gentle manual cleaning in water. The stems and roots were dried in an oven at 105 °C for four days prior to be weighed using a precision scale. All the leaves from each pot were also collected, dried and pooled because it was not possible to identify the precise sapling from which the leaves had fallen at harvest.

Concerning ants, at the beginning of the experiment, we manually collected, using entomology forceps, a series of 300 workers and 100 larvae from the 'reserve pots' and placed them in each of the 16 pots with ants (i.e. a total of 4800 ants and 1600 larvae at the beginning of the experiment) (**Fig. 1c**). To prevent workers from escaping, we regularly brushed paraffin oil on the inner face of the pots. A few minutes after installation, the workers tunnelled galleries and buried the larvae. Subsequently, in order to keep ants' activity in the pots, we added 100 workers and 100 larvae every two months as a surrogate of society with a queen regularly producing new nestmates. They were immediately accepted, and no aggressions were observed. Ant food, placed in small tubes to prevent it from spreading on the substrate, and watering troughs (i.e. water imbibing cotton disposed in small glass tubes), were provided three times per week in the PFA and FA treatments. Ant food was placed directly on the sediment surface in the pots without ants corresponding to the PF treatment with the same frequency and amount as for the PFA and FA treatments.

2.5 Sediments

At the end of the experiment in November, we used cylindrical, 30-cm-long PVC tubes (4 cm in diameter) to take 60 sediment cores from the experimental pots (i.e. two cores from six pots for treatments P, PF, PFA and FA and from three pots for S and SF), which were stored at -20 °C prior to chemical analyses.

The sediment cores were thawed by immersion in water at 70 °C for 2 min. The PVC tubes were then placed vertically to extract the sediment from the upper part of the vertical column (i.e. sediments closest to the surface to a depth of 12 cm: T [top] modality) and from the bottom of the pot (i.e. sediments closest to the bottom, to a depth between 12 and 24 cm: B [bottom] modality). Each sediment sample was homogeneously mixed before distribution into Erlenmeyer flasks.

To determine the acidity of the substrate, we conducted pH analyses by adding water (pH H₂O) and then KCl (pH KCl) to tear off the most acidic molecules of the substrate as they are not available by adding water. The analyses of the major ions for initial sediment conditions (**Table 1**) showed the concentration of conservative ions (sodium/calcium), which interact little with the plants, and the concentration of non-conservative ions (nutrients: nitrates, nitrites), which interact with the plants. The concentration of bio-available phosphorus (not released with water) was obtained using the Olsen method after adding NaCO₃ (Olsen et al. 1954). The concentration of total phosphorus was also measured, as well as the concentration of total organic carbon and total nitrogen. All the physicochemical analyses were determined at the *Plateforme d'Analyses Physico-Chimiques* (PAPC) from the *Laboratoire Écologie Fonctionnelle et Environnement* (LEFE, Univ. Toulouse UPS, INPT, CNRS).

Table 1 Major ions^a (in mg/L) in the initial sediment.

F ⁻	Cl ⁻	NO ₂ ⁻ -N	Br ⁻	NO ₃ ⁻ -N	SO ₄ ²⁻ -S	PO ₄ ³⁻ -P	Na ⁺	K ⁺	NH ₄ ⁺ -N	Mg ²⁺	Ca ²⁺
1.99	2.16	1.80	2.01	2.14	2.43	2.53	3.04	3.87	2.90	5.71	10.89

^a Nitrite values are given as nitrite nitrogen, nitrate values as nitrate nitrogen, ammonia as ammonia nitrogen, phosphate values as orthophosphate phosphorus, sulphate as sulphate sulphur.

2.6 Statistical analyses

The response of *P. nigra* cuttings to the different treatments was assessed via the sum of the length of branching of the stems ('branching length'), the dry weight of stems, dry weight of roots, dry weight of leaves (pooled by pot) and total biomass (i.e. dry weight of stems + dry weight of roots + dry weight of leaves) at harvest. The initial cuttings were not considered as part of the root system and were excluded from the final biomass. Total biomass was calculated at the cutting level, assigning one-sixth of the pot-pooled weight of leaves to each cutting, and at the pot level, summing the stem weight and the root weight of the six cuttings per pot before adding up the leaves' weight value. General linear mixed-effects models (LME; Zuur et al. 2009) and general linear models (GLM) were used in two steps. First, an LME with a maximum likelihood estimator (ML) was adjusted to each of the following four response variables (i.e. branching length, dry weight of stems, of roots and of total biomass), with the log-transformed covariates mean initial diameter and weight of the cuttings, and their interaction, selected as fixed factors by an AIC-based stepwise backward procedure. The sampling size (*n*) of these models was 144 (cuttings). Pots were nested within blocks as random effect. Two more LME models were run for dry weight of the leaves and for total biomass at pot level, where the *n* was 24 (pots), and blocks were the only random effect. The branching length, dry weights of stems, of roots and of total biomass (calculated at cutting level) were Box-Cox (lambda = 0.7), log + 1, log- and log-transformed to approximate their distribution to normality, respectively. Dry weight of leaves and total biomass at pot level did not need transformation. The residuals of the six LME models were used as dependent variables in 'full' GLMs with treatment (three levels: the three treatments with *P. nigra* cuttings present), genotype, and their interactions (the last two, only for the four response variables calculated at cutting level) as independent factors, and in 'reduced' GLMs for the four response variables calculated at cutting level with the same structure in an AIC-based backward selection procedure. Post hoc Tukey contrasts were used to explore the differences between all possible combinations of treatment and genotype for the four response variables at the cutting level, and between treatments for the two variables at the pot level.

The goodness-of-fit of all models was explored by subjecting them to a likelihood ratio test (i.e. compared to a null model, maintaining the same random structure in the case of LME models) using a $P < 0.05$ threshold (Pinheiro and Bates 2000; Bolker et al. 2009). We calculated the marginal (due to fixed factors) and conditional (due to fixed and random effects) adjusted r^2 for LME models, and the adjusted r^2 (hereafter, adj r^2) for the GLMs. In addition, we explored the distribution of residuals visually and with a Shapiro–Wilk’s test of normality ($P > 0.05$).

The variability of sediment chemical characteristics was summarized in a principal components analysis (PCA) after scaling all variables. Differences between experimental treatments and depths of the sediments sampled were explored with LME models using (1) the first two axes (Principal Components, PCs) as dependent variables, (2) treatment, depth, and their interaction as fixed factors and (3) pot nested within block as a random effect. The same procedure used for the vegetation variables was followed to assess the goodness-of-fit of the models. The comparison with control modalities (S and SF in Fig. 1c) allowed us to verify if changes were caused by ants, plants, ant food and their combinations.

All analyses were conducted using RStudio version 1.3.959 (RStudio Team 2020). LME models were run using the function *lme* of the package *nlme* (Pinheiro et al. 2019). The functions *stepAIC* and *r.squaredGLMM* of the package *MuMIn* (Barton 2009) were used to run the stepwise backward selections and calculate the marginal and conditional adjusted r^2 . Contrasts were run with the function *lsmeans* of the package *lsmeans* (Lenth 2016). The PCA was conducted with the function *rda* of the package *vegan* (Oksanen et al. 2019). Transformations were done using generic functions. Plots were obtained with the function *ggplot* of the package *ggplot2* (Wickham 2016).

3 Results

3.1 Growth of cuttings throughout the experiment

During the first month of the experiment, poplar cuttings grew consistently at a mean pace of ca. 5 cm per week, then stabilized their growth for the rest of the experiment to a steady growth of less than 2 cm per month, to reach ca. 40 cm at harvest (Fig. 2). The increase in total branch length from the beginning of the measurements to the end of the experiment varied across individuals between 6.7 and 59.6 cm (mean of 26.9 cm). However, no significant differences in growth over time were found between the three treatments (mixed models with time and treatment as fixed factors, not shown).

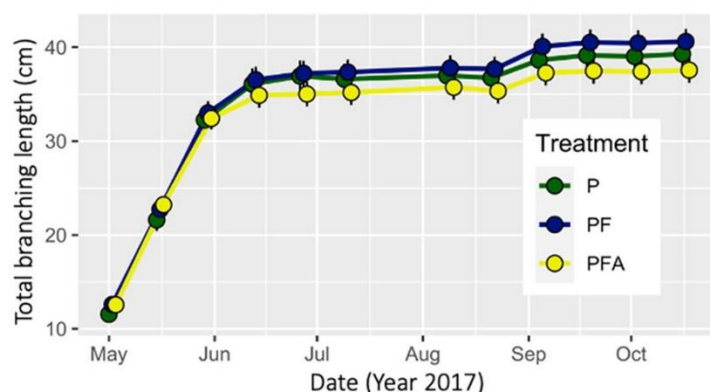


Fig. 2 Total branching length (including all stems and all branches) of the poplar cuttings over the course of the experiment. Each point represents the mean length \pm 1 SE of 48 cuttings per treatment. Treatment: ‘*P. nigra* alone’ (P); ‘*P. nigra* without ants and with ant food’ (PF); and ‘*P. nigra*, ants and ant food’ (PFA). No data were recorded on 25 July. Note that values of each treatment were slightly offset along axis X to improve visualization, but date of collection was the same.

At harvest, the dry weight of the stems ranged across individuals between 1.35 and 8.30 g (mean of 3.41 g), that of the leaves ranged between 3.58 and 7.19 g (mean of 5.32 g), and that of the roots varied between 0.04 and 3.47 g (mean of 0.64 g). Total biomass calculated per cutting ranged between 4.97 and 15.90 g (mean of 9.37 g). All these values are weight per cutting, but in the case of the leaves, the values were obtained by dividing the weight measured at each pot by six, and therefore, the range of the leaves and of total biomass, which used weight of leaves in the calculation, were underestimated.

3.2 Effect of initial cutting weight and diameter

There was a significant effect of initial mean diameter and initial cutting weight on stem branching length, dry weight of the stems, of the roots and total biomass at harvest, with 12.3%, 60.0%, 4.3% and 19.1% of the variability explained with the two covariates alone or combined, respectively (LME models, marginal adj r^2 values, $n = 144$ cuttings, **Table 2**; observed values in three top panels and bottom panel, **Fig. 3**). Mean diameter and initial cutting weight (both averaged by the six cuttings per pot) did not affect dry weight of the leaves and the former covariable explained 16.2% of total biomass when it was calculated at the pot level (LME models, marginal adj r^2 values, $n = 24$ pots, **Table 2**; observed values in two bottom panels, **Fig. 3**).

Larger cuttings, irrespective of the metric used to represent their size, led to longer branching length with larger above-, below-ground and total biomass at harvest (all panels **Fig. 3**, significant and positive model estimates of **Table 2** represented by blue lines).

Table 2 Estimates and diagnostics of LME models supporting relationships of initial mean diameter and initial cutting weight (fixed factors, including interaction, log transformed), and total branching length of the stems (length of all stems and length of all branches), dry weight of the stems, of the roots, of the leaves, and total biomass at harvest.

Variable	Transformation dependent variable	n	Intercept	Estimate initial mean diameter	Estimate initial weight	Estimate initial mean diameter : initial weight	l.r.t	R2m	R2c
Branching length (cm)	Box-Cox ($\lambda = 0.7$)	144	6.78 ± 1.36	Out	3.81 ± 0.85	Out	$P < 0.001$	0.123	0.158
Dry weight of stems (g)	log+1	144	-0.09 ± 0.34 (n.s.)	0.50 ± 0.27	0.36 ± 0.14	Out	$P < 0.001$	0.600	0.634
Dry weight of roots (g)	log	144	-8.68 ± 4.05	3.79 ± 2.40	5.12 ± 2.44	-2.43 ± 1.19	$P = 0.080$	0.043	0.197
Dry weight of leaves (g)	None	24	5.32 ± 0.25	Out	Out	Out	-	0	0.422
Dry weight of biomass (g)	log	144	1.75 ± 0.07	Out	0.30 ± 0.03	Out	$P < 0.001$	0.191	0.719
Dry weight of biomass (g)	None	24	-13.02 ± 9.09	11.41 ± 4.63	Out	Out	$P = 0.017$	0.162	0.543

See **Fig. 3** for observed values. Note that $n = 24$ for leaves, as we used averages of the six cuttings per pot for mean initial cutting diameter and initial weight in the models, but 144 values are represented in **Fig. 3d** and **i** as they are reported by cutting. For total biomass, models were run at the cutting level ($n = 144$) and at the pot level ($n = 24$), see Material and methods for calculations. l.r.t. likelihood ratio test; R2m marginal adjusted r^2 (due to fixed effects), R2c conditional adjusted r^2 (due to fixed and conditional effects); ':' denotes interaction.

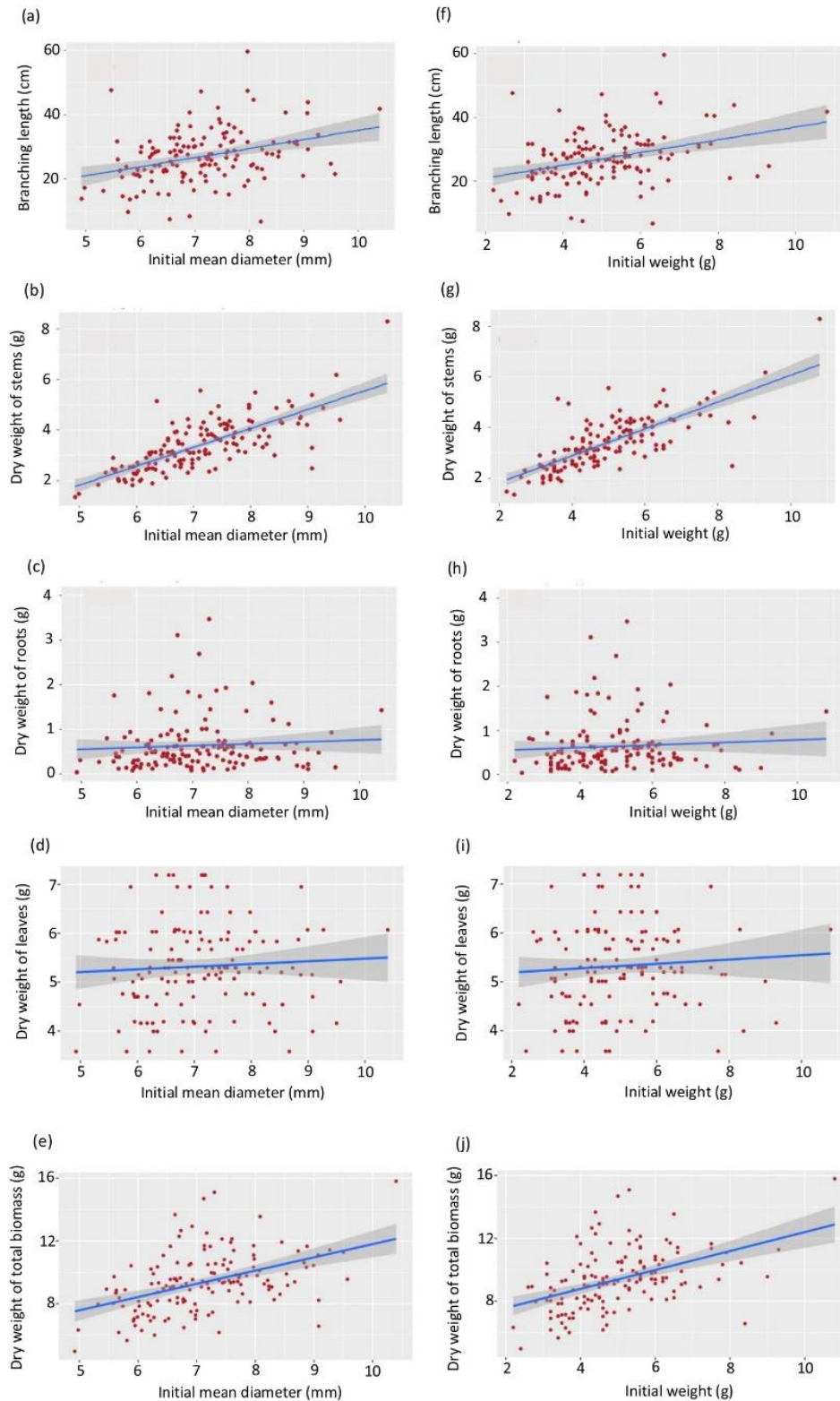


Fig. 3 Relationship of initial mean diameter (a–e) and initial cutting weight (f–j), and total length of the stems, dry weight of the stems, of the roots, of the leaves, and total biomass at harvest. Red points are observed values for the 144 individuals. Note that to calculate dry weight of leaves at the individual level, we divided the total weight of leaves collected at each pot by six and represented those estimated values in the graph (d and i). Blue lines with 95% confidence interval were obtained from a simple linear model between the response variable and the respective covariate, using the *geom_smooth* function of R package *ggplot2*, and are just depicted to indicate the direction of the relationships identified by the LME models, not predicted values.

3.3 Effect of experimental setup

The random structure of the pots and blocks explained 3.5% of the variability in branching length, 3.4% of dry weight of the stems, 15.4% of the roots and 52.8% of the total biomass of individual cuttings. The blocks explained 42.2% and 38.1% of the variability in dry weight of leaves and of total biomass per pot, respectively (differences between conditional and marginal adj r^2 values in **Table 2**).

3.4 Effect of treatment and genotype

Once the effects of the covariates and of the random structure of the experimental setup were accounted for, genotypes, treatments, and their interaction were selected by reduced models to explain differences in branching length, dry weight of stems, of roots and of total biomass among cuttings (observed values of residuals in **Fig. 4** showing contrasts among genotypes at each treatment, GLM model diagnostics in **Table 3**). Differences were in the order of about 10 cm in branching length, 0.6 g in dry weight of stems, 3 g in dry weight of roots and 0.4 g in total biomass. Genotype A and F produced larger plants compared to genotype E in the treatments ‘*P. nigra* alone’ (P) and ‘*P. nigra* without ants and with ant food’ (PF). Differences were found among treatments for dry weight of leaves and total biomass per pot (observed values of residuals in **Fig. 5** with contrasts among treatments, GLM model diagnostics in **Table 3**). The PF treatment produced significantly more total biomass than the other two treatments, i.e. P and ‘*P. nigra* with ants and ant food’ (PFA) and more leaf biomass than the PFA treatment. See predicted values of all GLM models in **Fig. SI1** (full) and **SI2** (reduced) in the Supplementary Information.

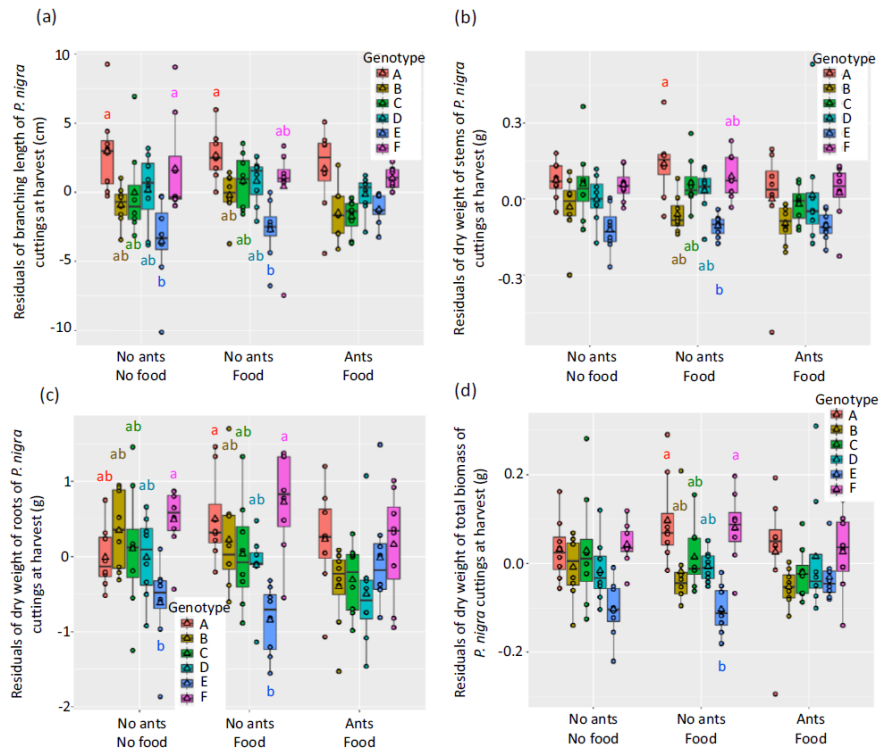


Fig. 4 Boxplots of residual (i.e. effects of initial mean diameter and initial weight removed, see Material and methods) trait values for each genotype and treatment of *P. nigra* cuttings at harvest: **a** branching length; **b** dry weight of stems; **c** dry weight of roots; and **d** dry weight of total biomass (i.e. stems + roots + leaves). First quartile to third quartile of observations are indicated with the median as a Black horizontal line. Whiskers indicate the maximum and minimum values within 1.5 the interquartile range (size of the box). Circles are the values of the eight pots. Triangles indicate mean values from the eight pots. Within each treatment, lowercase letters indicate homogeneous genotypes following ‘full’ GLM models (see Material and methods) and Tukey contrasts ($P < 0.05$). There were no differential responses across treatments within genotypes. Treatments are No ants No food: ‘*P. nigra* alone’ (P); No ants Food: ‘*P. nigra* without ants and with ant food’ (PF); and Ants Food ‘*P. nigra* with ants and ant food’ (PFA).

Table 3 Diagnostics of ‘full’ and ‘reduced’ GLM models supporting relationships of treatment, genotype or their interaction (explanatory factors), and total branching length of the stems (length of all stems and length of all branches), dry weight of the stems, of the roots, of the leaves, and total biomass at harvest.

Variable	Model	<i>n</i>	Explanatory factors	<i>F</i> value	d.f.	AIC	l.r.t	adjR2
Branching length (cm)	Full	144	Treatment : genotype	4.438	17, 126	673.3192	$P < 0.001$	0.290
	Reduced		Genotype	12.31	5, 138	663.7894	$P < 0.001$	0.283
Dry weight of stems (g)	Full	144	Treatment : genotype	3.338	17, 126	-190.8047	$P < 0.001$	0.218
	Reduced		Treatment + genotype	7.604	7, 136	-204.8319	$P < 0.001$	0.244
Dry weight of roots (g)	Full	144	Treatment : genotype	3.574	17, 126	288.2138	$P < 0.001$	0.234
	Reduced		Treatment : genotype	3.574	17, 126	288.2138	$P < 0.001$	0.234
Dry weight of leaves (g)	–	24	Treatment	4.700	2, 21	41.68931	$P = 0.021$	0.243
Dry weight of biomass (g)	Full	144	Treatment : genotype	3.075	17, 126	-281.7191	$P < 0.001$	0.198
	Reduced		Genotype	8.022	5, 138	-292.4898	$P < 0.001$	0.197
Dry weight of biomass (g)	–	24	Treatment	4.527	2, 21	60.62928	$P = 0.023$	0.235

See **Fig. 4** and **Fig. 5** for observed values, and **Fig. S11** and **S12**, Supplementary Information, for predicted values of full and reduced GLM models (see Material and methods for explanation). Note that $n = 24$ for leaves, as we used averages of the six cuttings per pot for mean initial cutting diameter and initial weight in the models. For total biomass, models were run at the cutting level ($n = 144$) and at the pot level ($n = 24$), see Material and methods for calculations. d.f. degrees of freedom, AIC Akaike Information Criterion, l.r.t. likelihood ratio test, adjR2 adjusted r^2 ; ‘:’ denotes interaction.

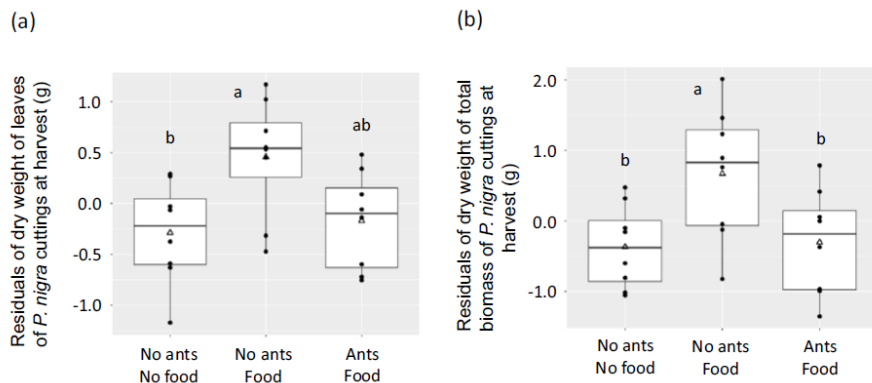


Fig. 5 Boxplots of residual (i.e. effects of initial mean diameter and initial weight removed, see Material and methods) trait values for each treatment of *P. nigra* cuttings at harvest: dry weight of leaves (left panel) and dry weight of total biomass (stems + roots + leaves, right panel). First quartile to third quartile of observations are indicated with the median as a black horizontal line. Whiskers indicate the maximum and minimum values within 1.5 the interquartile range (size of the box). Lowercase letters indicate homogeneous treatments after GLM models and post hoc Tukey tests ($P < 0.05$). Note that dry weight values are reported by cutting even though they were pooled by pot to be calculated and to run the GLM models and Tukey tests that provide statistical evidence. Treatments are No ants No food: ‘*P. nigra* alone’ (P); No ants Food: ‘*P. nigra* without ants and with ant food’ (PF); and Ants Food ‘*P. nigra* with ants and ant food’ (PFA).

3.5 Chemical properties of the sediment

The initial sediment (before the experiment) showed low N concentration (N = 0.06 mg for 47 mg of dry sediment) and other nutrients (**Table 1**). The pH values (pH H₂O = 8.45 and pH KCl = 7.74) remained stable during the experiment. At the end of the experiment, a total of 41% of variability in sediment chemical properties could be explained with two axes (PC1 and PC2, **Fig. 6**) that separated top from bottom layers

marginally, with more nutrients at the top of the pots, according to LME models (PC1: adjusted marginal $r^2 = 0.253$, P value of fixed factor ‘depth’ = 0.069; PC2: adjusted marginal $r^2 = 0.183$, P value of fixed factor ‘depth’ = 0.099; **Table S11** in Supplementary Information). Magnesium, nitrates, chloride, calcium, sulphate and fluoride tended to concentrate in the usually more alkaline top layer. A summary of the sediment chemical properties at the end of the experiment in each of the six treatments, divided into top and bottom layers, is presented in the Supplementary Information (**Fig. S13**).

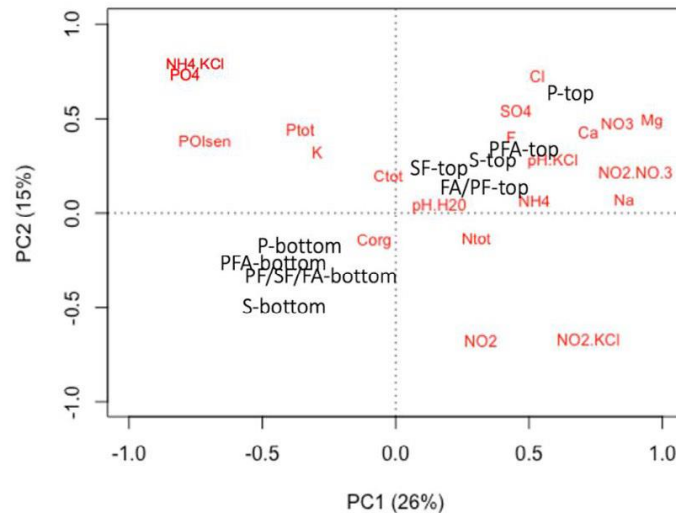


Fig. 6 Principal components analysis of sediment chemical properties at the top and at the bottom of the pots at the end of the experiment. Centroids of site scores (scaling = 2) for all plots belonging to different treatments, distinguished by depth levels, are depicted in black. Treatment: ‘*P. nigra* alone’ (P); ‘*P. nigra* without ants and with ant food’ (PF); ‘*P. nigra* with ants and ant food’ (PFA); ‘Sediment only’ (S); ‘Sediment only with ant food’ (SF); ‘Sediment only with ants and ant food’ (FA). Scores of soil properties (species scores, scaling = 2), in red.

There were no significant differences in the main two gradients of variability of sediment chemical properties between treatments at any of the two depths considered (P values of fixed factor ‘treatment’ and ‘treatment \times depth’ interaction > 0.7 for PC1 and PC2, **Table S11** in Supplementary Information). Thus, adding ant food (i.e. pots coded FA, PF, PFA and SF as opposed to those without ant food coded P and S) did not create statistically significant differences in the main two gradients of variability of the chemical properties of the substrate.

4 Discussion

4.1 Effects of ants on poplar growth

We found no effect of ants on cutting survival over one growing season in greenhouse conditions, as all cuttings survived to the end of the experiment. The 100% survival rate of cuttings confirms the ability of *Populus nigra* to regenerate from fragments on moist and open alluvial surfaces, as reported in the field on several European rivers (Barsoum 2002; Francis et al. 2004; Francis and Gurnell 2006). In addition, no significant effect of the treatments (‘*P. nigra* alone’ [P], ‘*P. nigra* without ants and with ant food’ [PF] and ‘*P. nigra* with ants and ant food’ [PFA]) could be found on growth of individual poplar cuttings, neither regarding the branching length nor the dry weight of stems, roots or total biomass (**Fig. 4**; **Table 3**). Different interpretations may explain this result. First, poplars are highly plastic (generalist genotypes) (Cronk 2005; Bush et al. 2021) which allows them to cope with a wide variety of biotic–abiotic environmental conditions (Viger et al. 2016; Liu and El-Kassaby 2019). Poplars may simply not respond to the presence of ants. Second, the cuttings used in this experiment, which represent a vegetative regeneration

modality and not a sexual regeneration from seeds, may have provided a high-energy reservoir for initial poplar growth on alluvial substrates with very poor nutrient content, which could have overridden any positive effect provided by ants. Thus, both the survival rate and growth patterns we observed suggest that poplar regeneration from fragments on alluvial bars does not require any facilitative effects from ants. Third, the physical restructuring of sediment by ants and nutrient accumulation that occurs in nature may not have lasted long enough (only one growing season) or have been too subtle in the pots to cause any effect on poplar growth. Finally, in the experiment, the young poplars did not undergo water stress, but such stress is very common on alluvial bars during the summer in temperate contexts and it can potentially affect the relationship between young poplars and ants. In short, poplars may respond to ants, but this has not been detected in this experiment.

Dry weight of leaves and total biomass, however, showed significant differences across treatments when data were pooled by pot (**Fig. 5; Table 3**), indicating increased poplar growth for the PF treatment, i.e. no ants but with the addition of ant food. We observed that the ant food we added directly to the sediment surface was rapidly decomposed by microorganisms and could thus be incorporated into the substrate during sprinklings and potentially made available for the poplars. In the P treatment no additional nutrients were added, and in the PFA treatment the added nutrients in form of ant food were systematically and rapidly consumed by the ants themselves and thus could not be directly exploited by the plants. Consequently, only in the PF treatment the plants could directly benefit from the additional nutrients, and this potentially resulted in the increased biomass we observed. If ant food was taken by poplars in the treatment PF, and nutrients were ingested by ants in the PFA, no statistical differences in relation to the concentration of nutrients in the sediment between the three treatments would be expected, as we indeed found. Finally, this observation suggests that ants potentially reduce the amount of resources on the sediment surface that would become available to young poplars. This interpretation requires to be validated with further experiments and field observations.

4.2 Variations in nutrient concentrations

McGinley et al. (1994) showed that the presence of ant nests encourages a higher activity of mineralization by microorganisms, and a change in pH, generally towards neutral, thus affecting the bioavailability of nutrients, in particular phosphorus. With a pH close to 7, the content of calcium and potassium cations is affected, involving positive changes in fertility and nutrient availability for seedling recruitment (Almeida et al. 2019). However, very low concentrations of nutrients in the sediments were found at the end of the experiment in each pot (**Fig. SI3** in Supplementary Information). This experiment highlights all the difficulties to correctly reproduce the natural context of nutrient flow in ex situ conditions. In nature, ant nests represent long-term nutrient hotspots for plants because they function as open systems connected to a large foraging area. We observed no nutrient accumulation in the treatments with ants probably because ants were processing the provided ant food in a very restricted area, and the quantity that was processed through ingestion may not have been enough to induce a significant positive effect on nutrient concentration and subsequent poplar growth. The small variation in nutrient concentrations we observed between pots could have also resulted from the low number of ants in the pots (300 at the beginning of the experiment) and their activity compared to natural conditions where nests harbour thousands of individuals and a queen in full activity. Rasse and Deneubourg (2001) showed a positive correlation between nest volume and the number of *Lasius niger* individuals, and the digging rate and resulting impact on the sediment. Buhl et al. (2004) also showed experimentally, with *Messor sanctus* (Emery, 1921), that the final volume of excavated sand is positively correlated with the number of ants. It is likely that chemical changes related to ants are also proportional to their number and their biomechanical activity.

Significant differences between depths for the different elements were observed. The repeated sprinklings within the pots may have resulted in lixiviation outside of the pots and this impeded nutrient accumulation. Furthermore, our observation at harvest indicated larger development of the fine roots of the poplars at the bottom of the pots. Nutrient uptake by plants was thus presumably concentrated at the bottom of the pots

and probably also led to a reduction in the concentration of the elements there. At the same time, evaporation may have concentrated a part of the elements at the top of the pots where the roots did not concentrate. An additional explanation could be the bioturbation activity of ants that translocated sediment from the bottom to the top of the pots and helped define a zone of nutrient accumulation near the surface. It should be noted that ant food was provided in small tubes in the PFA and FA treatments to prevent it from leaking into the substrate. Leakage occurred in the PF treatment, which resulted in greater biomass production, especially leaves, in the plants. PF treatment was supplemented with nutrients in a viscous form (and perhaps slower-release), whereas in the PFA and FA treatments, the ants processed the food. In the PFA treatment, food was excreted within the 'nest' structures as faeces (liquid) that could be more easily moved to the bottom of the pots and lixiviated.

4.3 Genotype effect

Given the variability and stochasticity of habitat conditions on alluvial bars subjected to a hydrological disturbance regime, black poplars have maintained a large genetic diversity that generates differential phenotypic responses to the physical environment ([Viger et al. 2016](#); [Garavillon-Tournayre et al. 2017](#); [Hortobágyi et al. 2018](#)). Within several treatments, the different genotypes showed significant growth differences (**Fig. 4**). The largest differences appeared most of the time between E, A and F genotypes with smaller plants for E and bigger plants for A and F. Furthermore, the initial weight and size of the cutting also positively affected growth. Combined together, these results indicated that the total biomass of poplars after the first growing season is positively affected by the initial size of the fragments and is modulated by the genotype independently from the treatments. The genotypic effect on poplar growth identified in this experiment suggests that genetic factors of the plants (and possibly the ants) may also potentially affect the type and quality of their relationship with the ants.

4.4 Lessons and perspectives

The significant response (at the pot level) between the presence or absence of ants was driven by leaf biomass (i.e. the differences in total biomass was driven mainly by leaf biomass and the observed differences fade out when total biomass was expressed at the cutting level). The leaf compartment is thus the most responsive to differences in nutrient levels in the soil and thus, it may be the first one to target in the search for ant effects.

Seedlings originating from seeds are more sensitive than cuttings to slight variations in the physical environment during the first year of growth ([Mahoney and Rood 1998](#); [Guilloy-Froget et al. 2002](#); [Stella and Battles 2010](#); [Guilloy et al. 2011](#)). Consequently, new experiments using *P. nigra* seeds and seedlings instead of cuttings might be more informative to test the effect of ants on seedling survival and initial growth. However, the use of seeds in experiments should be tested first because under controlled conditions, germinated seeds could be at the very beginning of their growth uprooted by ants translocating sediment particles. The action of digging galleries along the initial roots could also cause the seedlings to suffer in their very early stage of recruitment from mechanical and water stress. Thus, it is probably best to add the ants to the pots after a few days (to be defined by preliminary tests) following the germination of the plants.

In addition, the survival of the ant colony at the end of the experiments should be assessed. Variation in the survival rate of ants in the pots could explain some of the variation in the growth response of poplars. We suggest that ant activity and abundance in the pots should be monitored and quantified, respectively, during the experiment and at the end of the experiment. Ant abundance at the end of the experiment can be quantified by the complete extraction of ants from the pots by heat or by mechanical sorting techniques such as flotation in water after sand removal ([Aribas et al. 2016](#)). From a functional point of view, ant activity on the surface of the sediment can be monitored by observers ([Cole et al. 2010](#)) or by automatic camera tracking (e.g. [Balch et al. 2001](#); [Imirzian et al. 2018](#)). Within the sediment column, the description

and quantification of both gallery networks and particle transfers by ants can be done using complementary X-ray tomography and tracers ([Capowiez et al. 2021](#)).

We suggest two complementary recommendations in future experiments: (i) use the largest possible array of genotypes to prevent idiosyncratic response of a single *P. nigra* genotype to the presence of ants; (ii) the number of pot replicates should be reduced in alternative experiments to be able to introduce a full colony of thousands of ants with its queen in activity in fewer bigger pots. Ex situ experiments should be combined with in situ observations of poplar survival and growth patterns with and without the occurrence of ant nests. Our field observations on the Allier and Garonne Rivers, France, indicate that ant nests are often located at the foot or in the vicinity of young poplar individuals. Additionally, the survival and growth of poplar seedlings and saplings with and without ant nests should be monitored over time. Some studies have shown that the structure of riparian forests affects ants, for example, by mediating their trophic breadth ([García-Martínez et al. 2017](#); [Zina et al. 2021](#)). Besides measuring a potential effect of ants on poplar seedlings, we also need to explore the possibility of a collaboration (i.e. mutualism) between the taxa. On the one hand, and despite the results of the present study, ant activity may favour poplar establishment under certain conditions and maybe for specific genotypes. On the other hand, the stabilization of the alluvial bar substrate during floods by the root system of poplars may provide stable micro-habitats for ants in an area generally subject to submersion and erosion during flooding. For example, it would be interesting to investigate whether the founding queens chose to dig their first gallery in the immediate vicinity of poplar seedlings in a context exposed to flooding. Established poplars can also play the role of aerial refuge habitats for ants during prolonged submersions of the alluvial bar (i.e. avoidance strategy). By providing resistance to flow, poplars also trap large amounts of fine sediment, organic matter and seeds during flooding ([Goodson et al. 2001](#); [Gurnell et al. 2006](#); [Corenblit et al. 2009](#); [Erskine et al. 2012](#); [Manners et al. 2015](#); [Corenblit et al. 2016b](#); [Diehl et al. 2017](#)). This local accumulation of mineral and organic material can ultimately be beneficial to ants. Poplars could also provide a local source of preys or other food sources (e.g. aphids) for ants ([Villar and Forestier 2009](#)). Another factor not present in the ex situ experiment is the effect of herbivores on poplar growth and how ants affect this effect. Ants are known to be frequent visitors of trees producing extrafloral nectar such as poplars ([Escalante-Pérez et al. 2012](#)). The predatory and aggressive actions of ants towards herbivores can significantly reduce the damage they cause on plant leaves ([Chaudhary et al. 2018](#)). No herbivores were detected in the greenhouse, so this potentially beneficial effect of ants as natural defenders of poplars could not be observed. The hypothesis of cooperation should be tested in situ by surveying the occurrence and activity of ants on young poplars and poplar growth over a few consecutive years. The challenging aspect of the analysis will be monitoring ant activity during flooding. The use of ant traps disposed in trees before a flood would be a way to proceed during natural events (e.g. [Blüthgen et al. 2000](#)). The effect of a flood on ants can also be simulated in situ and ex situ by derivation of the water flow towards a nest. Such manipulation would allow surveying ant dynamics during submersion.

5 Concluding remarks

Our experiment did not allow us to confirm our initial hypothesis, i.e. a positive effect of ant activity on poplar survival and growth. This leads to two possible conclusions. (i) Ants do not have any positive effect on poplar individuals, which, however, does not exclude that poplars may have positive effects on ants (e.g. providing a stable habitat or aerial refuge habitats during flooding in riparian ecosystems). (ii) The design of our ex situ greenhouse experiment (e.g. a limited number of ants in one pot; the use of cuttings and not of seedlings) did not reveal positive effects of ants on poplar survival and growth, which could possibly occur in situ within dynamic riparian ecosystems.

This experiment involved mastering the logistics to manipulate two living organisms from the kingdoms of flora and fauna, one sessile and one mobile. We provided here suggestions to improve future in situ and ex situ experiments and field measurements exploring potential mutual or one-way interactions between ants and poplars in riparian ecosystems.

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Author contributions

DC, BC, RC, AD, VGG, FG, EGS, FJ, LL, LM, TO, JS, ET and ITB designed and carried out the experiment and wrote the paper; PD, CM and YT provided support for lab analyses and statistics. PD and EGS conducted all the statistical analyses with RStudio. All authors read and approved the final manuscript.

Declarations

Competing interest. No specific funding was received for conducting this prospective study.

Ethical approval. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Data availability. The datasets generated during and/or analysed during the current study are available on Zenodo.

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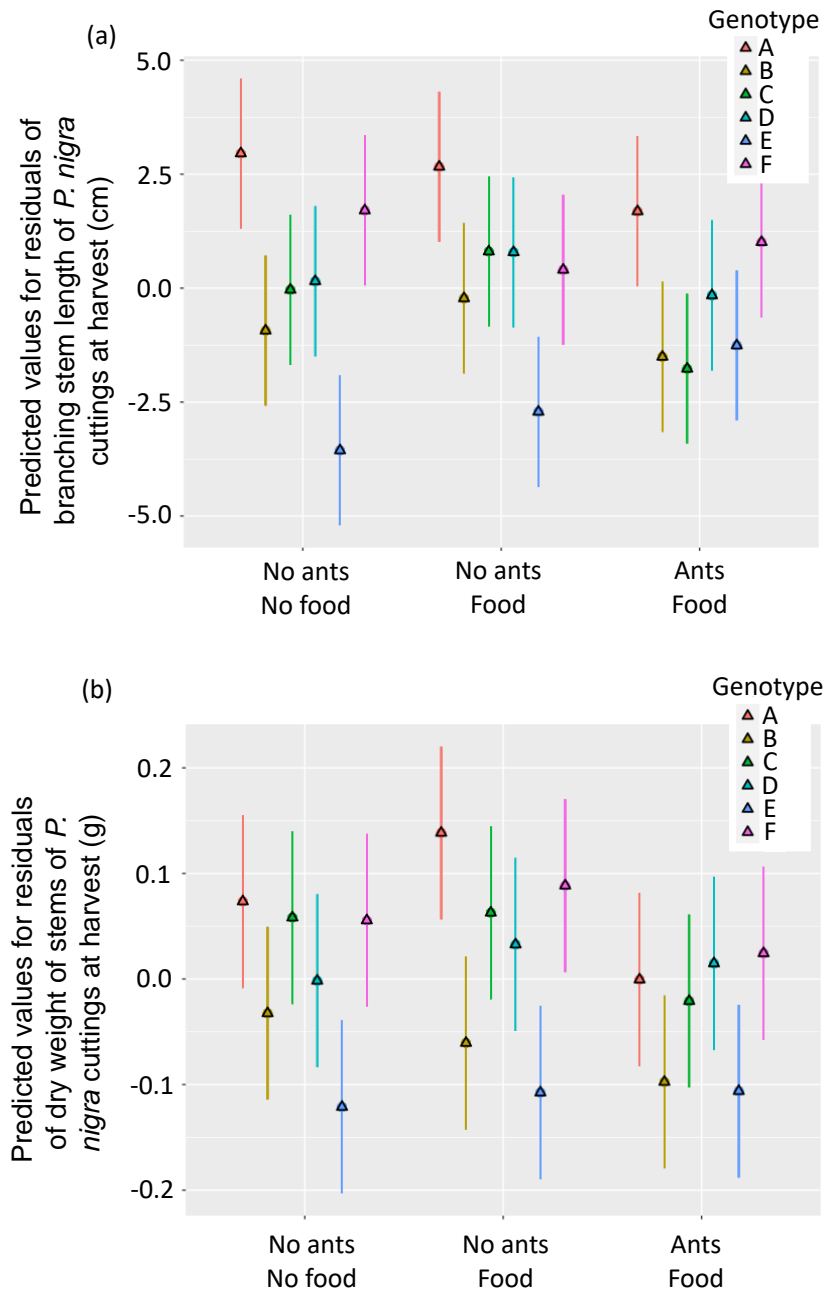
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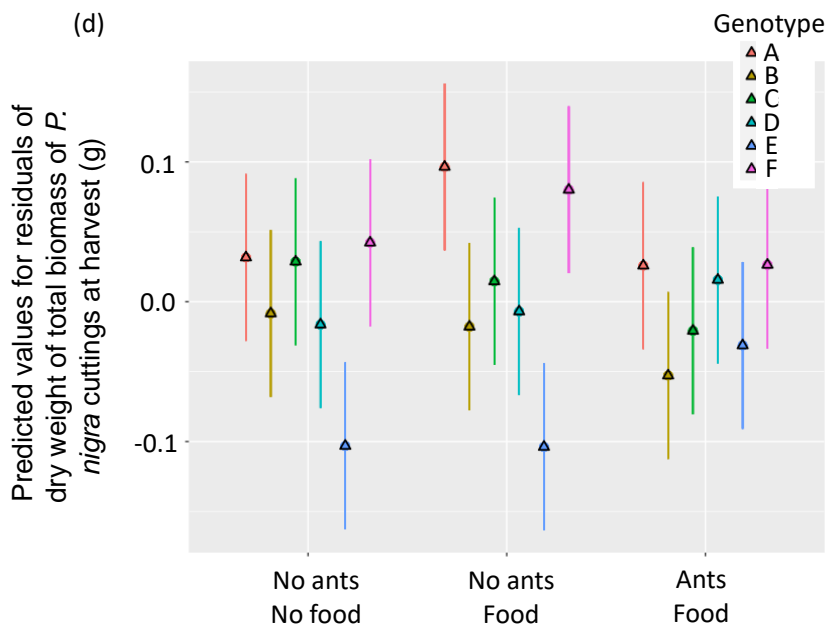
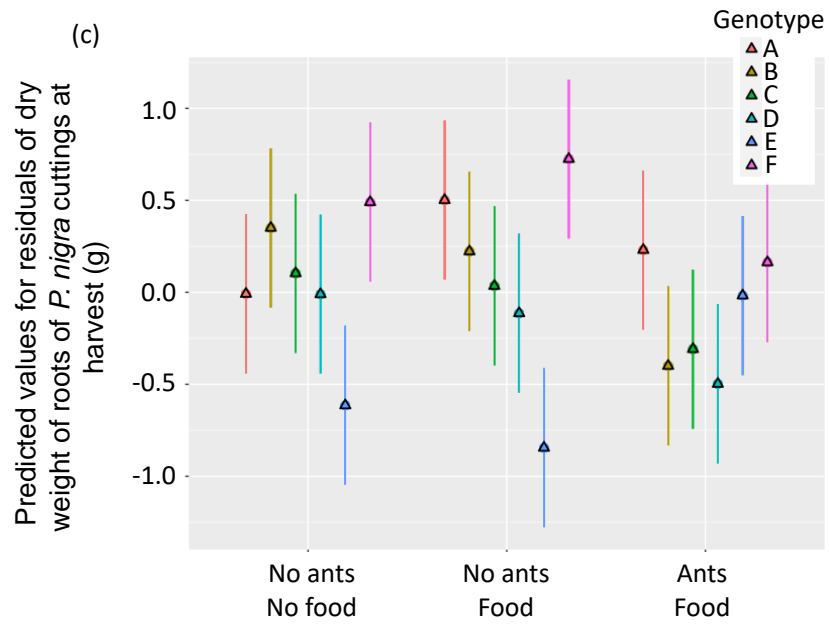
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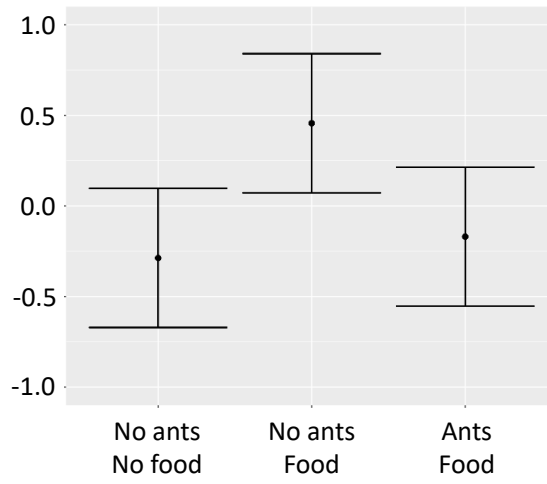
Supplementary Information

Fig. S11 Predicted values with 95% confidence interval of ‘full’ GLM models with treatment (three levels: the three treatments with *Populus nigra* cuttings present), genotype, and their interactions as independent factors for residuals of branching length, dry weight of stems, roots, and total biomass, calculated at the cutting level. Contrasts of full GLM models are shown in **Fig. 4** and **Fig. 5**. Only treatment as factor for residuals of dry weight of the leaves and total biomass when calculated at the pot level (see Methods and Results sections for more details). (a) branching length, (b) dry weight of the stems, (c) dry weight of the roots, (d) total biomass at cutting level, (e) dry weight of leaves, (f) total biomass at pot level. See model diagnostics in **Table 3**.





(e)
Predicted values for residuals
of dry weight of leaves of *P.*
nigra cuttings at harvest (g)



(f)
Predicted values for residuals
of dry weight of total biomass of
P. nigra cuttings at harvest (g)

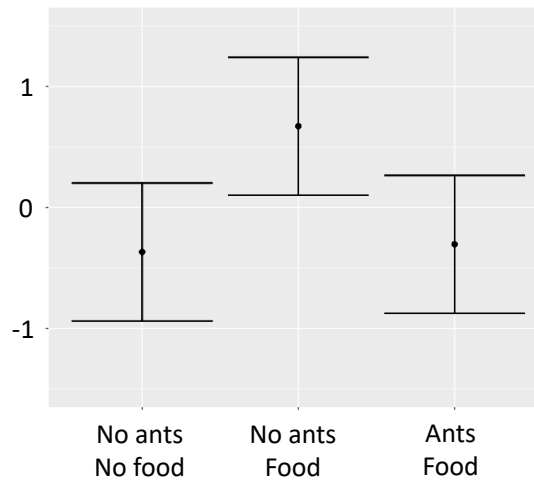
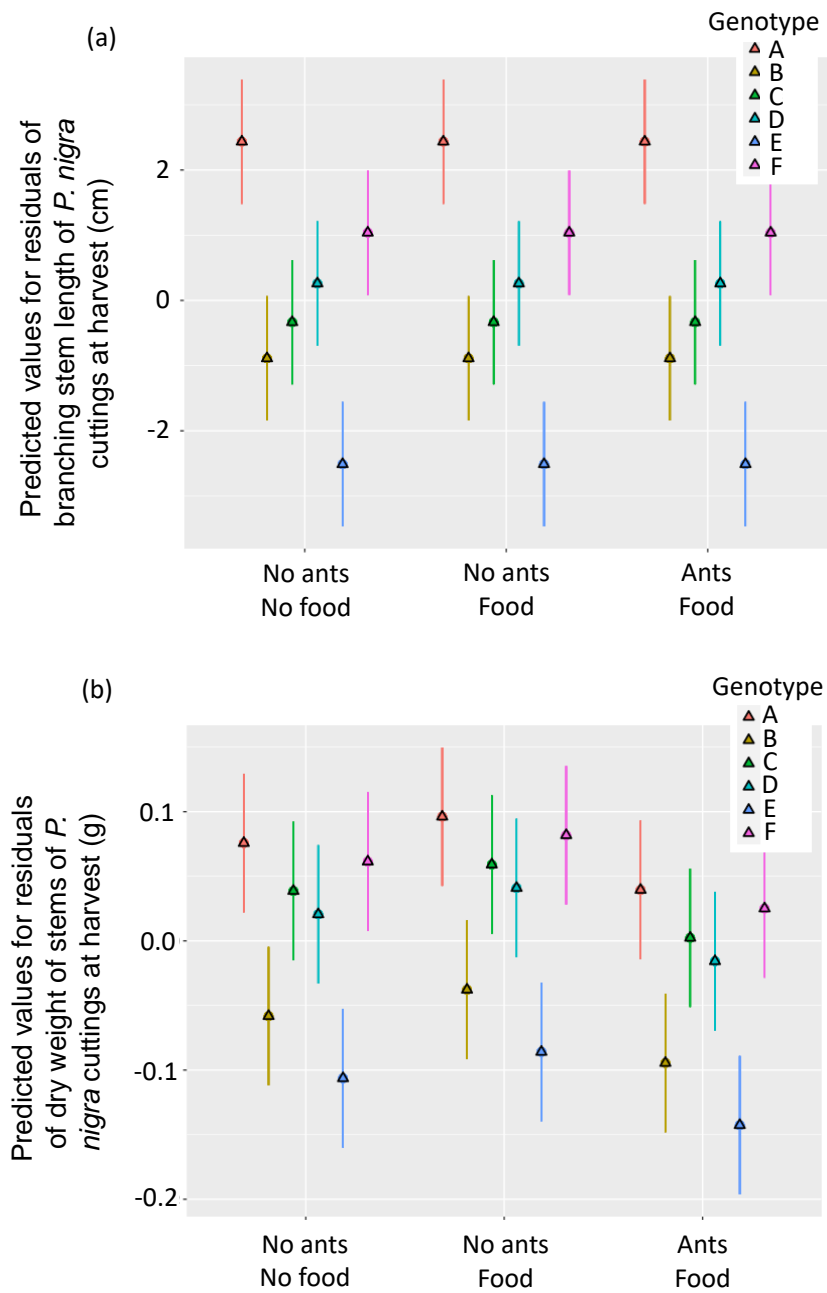


Fig. SI2 Predicted values with 95% confidence interval of ‘reduced’ GLM models with treatment (three levels: the three treatments with *Populus nigra* cuttings present), genotype, and their interactions as independent factors for residuals of branching length, dry weight of stems, roots, and total biomass, calculated at the cutting level. See model diagnostics in **Table 3**.



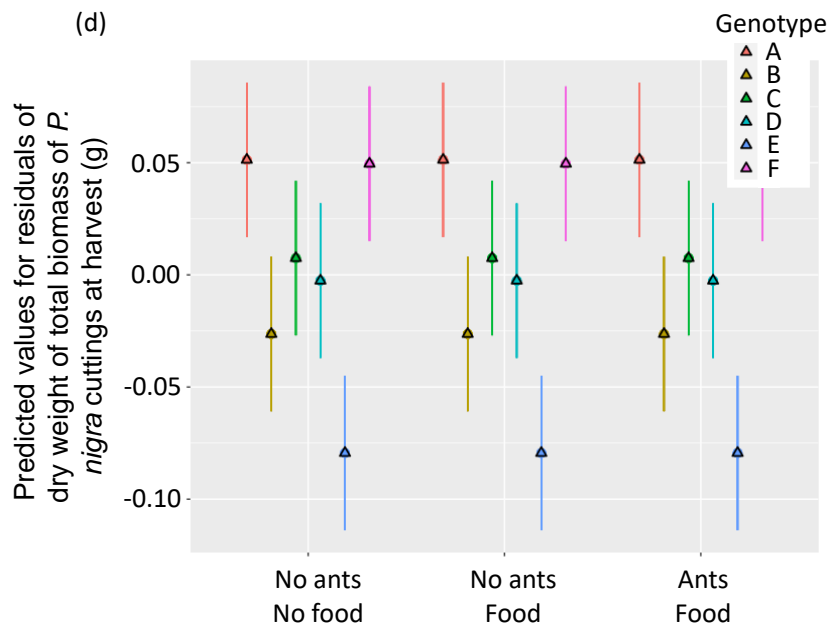
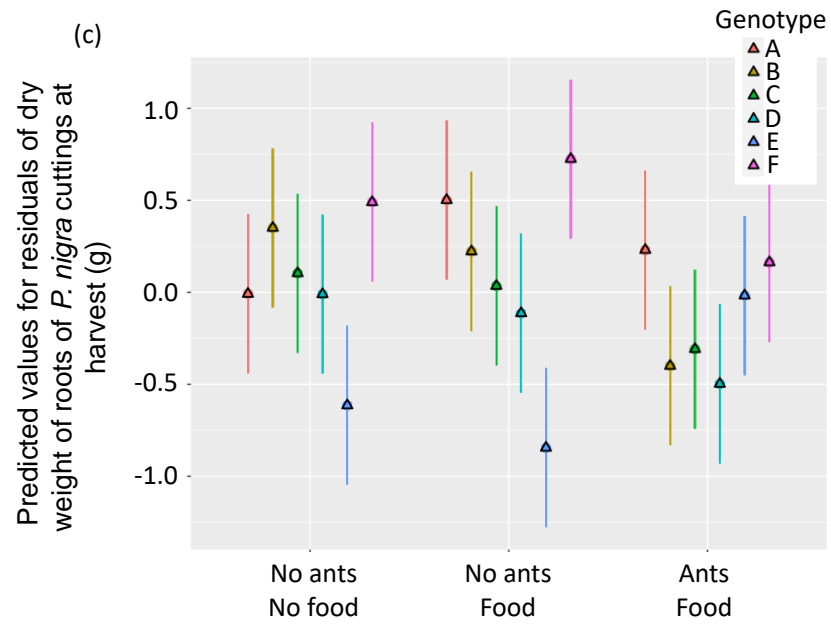


Fig. S13 Mean \pm 1 SE of sediment chemical properties. FA – food, ants, without *P. nigra* cuttings, P – *P. nigra* cuttings, no ants, no food, PF – *P. nigra* cuttings, food, no ants, PFA – *P. nigra* cuttings, food, ants, S – Control, SF – no *P. nigra* cuttings, food, no ants. B – Bottom of pot. T – Top of pot.

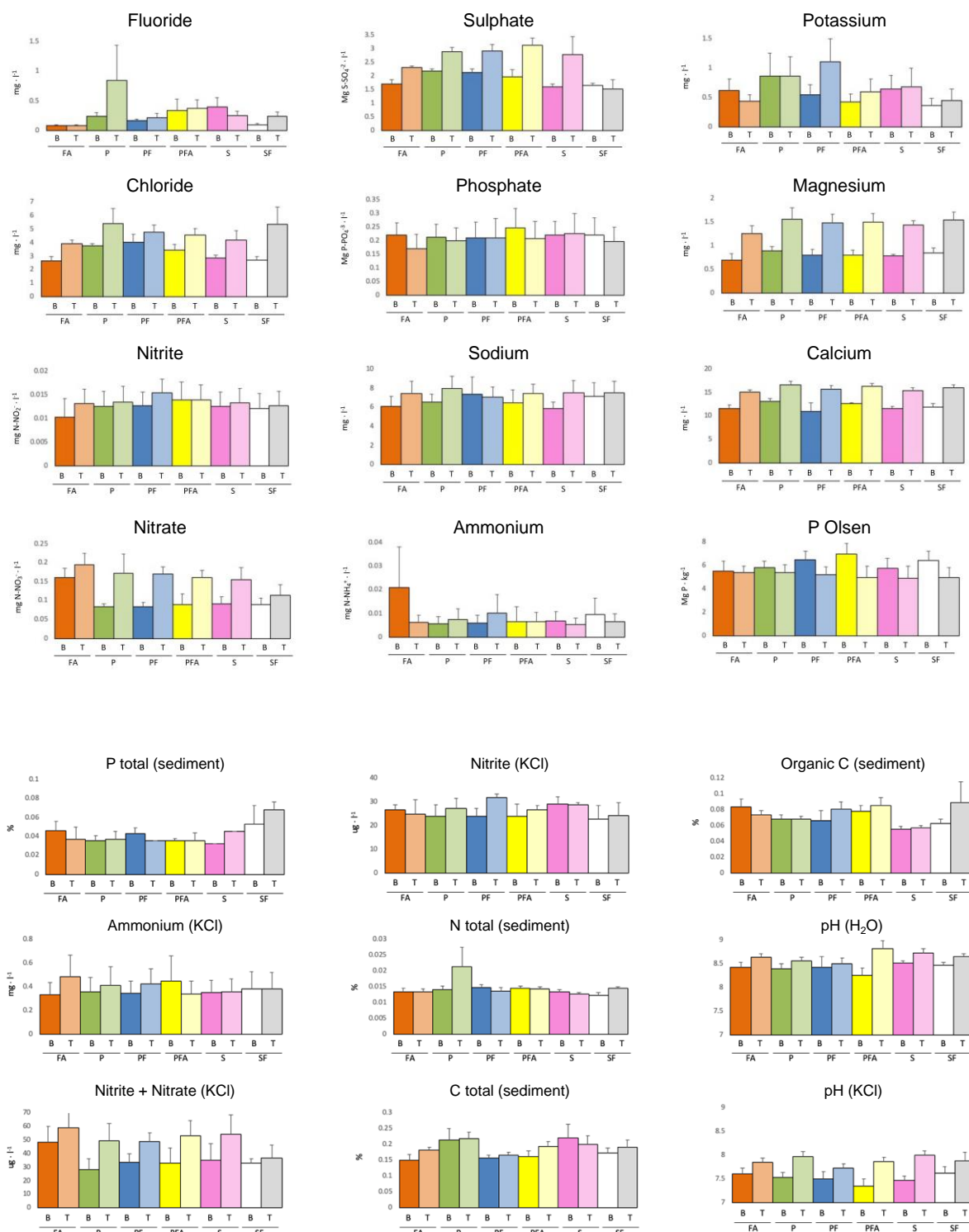


Table S11 Diagnostics of LME models supporting relationships of principal components 1 and 2 summarizing sediment chemical characteristics with fixed factors depth of the pot where sediment samples were taken (two levels: top and bottom), and treatment (six levels: P - *P. nigra*, PF - *P. nigra* + ant food, PFA - *P. nigra* + ant food + ants, FA - ant food + ants, S - control, SF - ant food). Random effects were pot nested within block. See **Fig. 6** for species scores (chemical characteristics) and site scores (centroids of each treatment and depth) along PC1 and PC2. l.r.t. = likelihood ratio test; R2m = Marginal adjusted r^2 (due to fixed effects); R2c = Conditional adjusted r^2 (due to fixed and conditional effects).

PC	Fixed factor	numDF	denDF	Fvalue	Pvalue	l.r.t	R2m	R2c
PC1	Treatment	5	8	0.0485	0.998	$P < 0.001$	0.253	0.789
	Depth	1	36	3.5151	0.069			
	Treatment : depth	5	36	0.5402	0.745			
PC2	Treatment	5	8	0.0630	0.996	$P = 0.003$	0.183	0.766
	Depth	1	36	2.8674	0.099			
	Treatment : depth	5	36	0.3032	0.908			

R Script SI1. Example of R scripts for LME and GLM models: Dry weight of stems

Description of objects:

PS_tige: A vector of 144 elements containing dry weight of the 144 cuttings, continuous variable

P_initial: A vector of 144 elements containing the initial fresh weight of the 144 cuttings, continuous variable

DiamMoy_initial: A vector of 144 elements containing the initial mean diameter of the 144 cuttings, continuous variable

Bloc: A vector of 144 elements containing the block of pots in the experimental design, factor variable

Pot: A vector of 144 elements containing a unique pot identifier, factor variable

popant_data: A data frame of dimensions 144 x 13 containing all measurements of the experiment at the cutting level

Tr: A vector of 144 elements containing the treatments (3 levels), factor variable

PM: A vector of 144 elements containing the genotype (6 levels), factor variable

R scripts:

```
## y = PS_tige (dry weight of stems), cutting level
```

```
#step 1 LME - see Table 2 of paper
```

```
covar_PStigereg3 = lme((log1p(PS_tige)~(log(P_initial))*(log(DiamMoy_initial))),  
random=~1 | Bloc/Pot,data=popant_data, method="ML")
```

```
step_covar_PStigereg3 = stepAIC (covar_PStigereg3, .~, random=~1 | Bloc/Pot, method="ML",  
data=popant_data, direction="backward", trace=TRUE, steps=10000)
```

```
final_covar_PStigereg3 = lme(log1p(PS_tige)~log(P_initial)+log(DiamMoy_initial), random=~1 | Bloc/Pot,  
method="ML", data=popant_data)
```

```
PStigereg3_res = residuals (final_covar_PStigereg3)
```

```
Hist (PStigereg3_res)
```

```
shapiro.test (PStigereg3_res)
```

```
summary (final_covar_PStigereg3); anova (final_covar_PStigereg3, type="marginal"); r.squaredGLMM  
(final_covar_PStigereg3)
```

```
null_covar_PStigereg3 = lme (log1p(PS_tige)~1, random=~1 | Bloc/Pot, data=popant_data, method="ML")
```

```
anova (final_covar_PStigereg3, null_covar_PStigereg3) ###likelihood ratio test
```

```
#step 2 GLM - see Table 3 of paper
```

```
PStigereg3_reg3.full = lm (PStigereg3_res~Tr*PM)
```

```
step_PStigereg3_reg3 = stepAIC (PStigereg3_reg3.full, .~, data = popant_data, direction="backward",  
trace=TRUE, steps=10000)
```

```
PStigereg3_reg3.reduced = lm (PStigereg3_res~Tr+PM, data = popant_data)
```

```
PStigereg3_reg3.null = lm (PStigereg3_res~1, data=popant_data)
```

```
(aov_PStigereg3_reg3.full = anova (PStigereg3_reg3.full))
```

```
(aov_PStigereg3_reg3.reduced = anova (PStigereg3_reg3.reduced))
```

```
anova (PStigereg3_reg3.full, PStigereg3_reg3.null) ###likelihood ratio test
```

```
anova (PStigereg3_reg3.reduced, PStigereg3_reg3.null) ###likelihood ratio test
```

```
(leastsquare_PStigereg3_reg3.full = lsmeans(PStigereg3_reg3.full, pairwise ~ Tr*PM, adjust="tukey"))
```

```
###contrasts for Fig. 4
```

```
(leastsquare_PStigereg3_reg3.reduced = lsmeans(PStigereg3_reg3.reduced, pairwise ~ Tr, adjust="tukey"))
```

```
###contrasts not shown in the paper
```

```
(leastsquare_PStigereg3_reg3.reduced = lsmeans(PStigereg3_reg3.reduced, pairwise ~ PM, adjust="tukey"))
```

```
###contrasts not shown in the paper
```

```
Summary (PStigereg3_reg3.full)
```

```
AIC (PStigereg3_reg3.full)
```

```
BIC (PStigereg3_reg3.full)
```

```
logLik (PStigeregres_reg3.full, REML=FALSE)
plot (PStigeregres_reg3.full)
plotNormalHistogram (residuals (PStigeregres_reg3.full))
shapiro.test (residuals (PStigeregres_reg3.full))

summary (PStigeregres_reg3.reduced)
AIC (PStigeregres_reg3.reduced)
BIC (PStigeregres_reg3.reduced)
logLik (PStigeregres_reg3.reduced, REML=FALSE)
plot (PStigeregres_reg3.reduced)
plotNormalHistogram (residuals (PStigeregres_reg3.reduced))
shapiro.test (residuals (PStigeregres_reg3.reduced))

#
```