Soil origin impacts *Acacia longifolia* above and belowground development: water and nutrition as players

Running title: Soil impacts *Acacia longifolia* early development

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Abstract

Context: *Acacia longifolia* is an aggressive invader, disrupting habitats and communities worldwide. Understanding what drives its expansion is of paramount importance. Key functional traits include fast growth and the presence of bacterial symbionts performing nitrogen fixation.

Aims: To address early plant development of *Acacia longifolia* under different soils and growth conditions (water and nutrition availability), establishing the key factors that influence above-belowground responses.

Methods: Plants were grown in pots with soils collected from forest, agricultural and dune areas in relative proximity, in a controlled experiment designed to study water effect and nutrient availability on early growth. Growth parameters included shoot and root length, nodule number and weight. Bacteria were isolated from nodules for genetic diversity evaluation. Photosynthetic pigments and isotopic nitrogen and carbon analyses were performed to address nitrogen fixation and photosynthesis.

Key Results: Soil origin influenced plant growth and bacterial diversity. Largest plant development was achieved in forest soils with added nutrition and water. Plants from agricultural soil displayed higher aboveground development, however belowground nodule number and bacterial biodiversity decreased, maybe due to anthropogenic activities. Forest soil promoted belowground development and bacterial diversity. In dune soils overall growth was lower and nitrogen fixation was higher.

Conclusion: Abiotic factors influenced juvenile acacia development: nutrition acts as a growth enhancer, and soil origin, including its microbial communities, can be considered a development modulator. Bacterial diversity varied according to soil type.

Implications: Several above and belowground interactions showed the need for an integrative perspective to understand acacias invasive potential.

Keywords: *Acacia longifolia*, wattles, abiotic factors, nodulation, nitrogen fixation, plant-soil feedbacks, aboveground interactions, belowground interactions.

Introduction

The introduction of exotic plants is one of the major problems for local biodiversity, as it threatens the existence of endemic species as well as the integrity of flora and ecosystem communities. Invasion by alien plant species has human and economic impacts, affecting agriculture and forestry (Richardson and Rejmánek 2011; Kumar Rai and Singh 2020). Species from the leguminous *Acacia* genus are amongst the most aggressive invaders worldwide (Brockwell *et al.* 2005; Richardson and Rejmánek 2011; Richardson *et al.* 2015; Vieites-Blanco and González-Prieto 2020) causing severe problems in the habitats, including drastic changes in both above and belowground communities, as well as changes in the soil carbon and nitrogen reservoirs, and in the water cycle (Brockwell *et al.* 2005; Marchante *et al.* 2015; Duarte 2016). An important competitive advantage of most acacias in comparison to native plants results from the possibility of establishing mutualisms with nitrogen fixing bacteria present in the soil, thus promoting their own nutrition and growth (Marchante *et al.* 2015; Ulm *et al.* 2017a, b). Native legumes can be herbaceous or shrubs, and *A. longifolia* always presents competitive advantage considering many traits such as phosphorus acquisition, soil organic matter, vegetative growth (Fernandes *et al.* 2015) and a persistent seed bank (Ulm *et al.* 2017a, b). Therefore, the introduction of *Acacia* diminishes biological diversity and alters the functioning of ecosystems, unbalancing it and causing severe damage to their functionality (Hellmann *et al.* 2011; Rascher *et al.* 2011a; Marchante *et al.* 2015; Ulm *et al.* 2017a, b).

In Portugal, *Acacia longifolia* (Andrews) Willd., also known as Sydney Golden Wattle, is an important plant invader (Vicente *et al.* 2018). This species originates from southeast mainland Australia and Tasmania, and the first official record of introduction in the coastal areas of Portugal is in 1897 (Fernandes 2008, Carruthers *et al.* 2011, Kull *et al.* 2011) with the aim of stabilizing the dunes and controlling their erosion (Marchante *et al.* 2008, Vicente *et al.* 2018). Following introduction, *A. longifolia* has spread to adjacent environments and currently it is present in most of the Portuguese territory, including forest and agricultural areas. In what concerns forest areas,

A. longifolia invades both pine (*Pinus pinaster* and *P. pinea*), eucalypts (*Eucalyptus globulus*) and cork oak (*Quercus suber*) stands, decreasing productivity. Furthermore, forest certification nowadays requires the existence of a control plan for invasive control. On agricultural systems, the control of acacias in areas surrounding production areas must be performed, otherwise major losses of productivity will occur.

Acacia longifolia alters nutrient cycles, water availability and community composition, and might even disrupt fire regimes (Marchante *et al.* 2008; Rascher *et al.* 2009; Hellmann *et al.* 2011; Le Maitre *et al.* 2011). This species can be considered an ecosystem engineer and produces a high number of seeds (Richardson *et al.* 2000; Hellmann *et al.* 2011; Marchante *et al.* 2011). Furthermore, its fast growth rate and the presence of evergreen phyllodes lead to the formation of dense canopies (Le Maitre *et al.* 2011), which limit the availability of light to understory plants (Rascher *et al.* 2011a; Souza-Alonso *et al.* 2017), affecting species growing underneath and reducing species' diversity. In addition, *A. longifolia* also has shape plasticity, adapting to available height class niches by adopting shrub or small tree forms (Rascher *et al.* 2011a, b). The invasive success of this species is also due to the strategies adopted by the plant during the periods of drought. These consist in morphological adaptations of the phyllodes and adjustments in the water uptake to reduce water losses, as well as the development of extensive root systems allowing to use water sources from deeper soil layers in comparison to native flora (Antunes *et al.* 2018). Furthermore, an accumulation of litter is frequently observed under the canopy of *Acacia* species due to their high leaf turnover rate (Zhang *et al.* 2020), which contributes to the alteration of soil characteristics and nutrient cycles (Hamad-Sheip *et al.* 2021). Biomass accumulation increases organic matter content as well as nitrogen (N) and carbon (C) pools. While at first this will benefit native species, local flora cannot compete with acacias growth rates that gradually take over their habitats. The changes in soil properties attributed to *A. longifolia* includes pH and N, phosphorus (P) and C pools, as well as the N/P balance, progressively disrupting nutrient dynamics, water cycle and soil microbial communities (Hellmann *et al.* 2011; Souza-Alonzo *et al.* 2014, 2017; Ulm *et al.* 2017a). Several studies have also showed that plant

invasions may cause an alteration of the soil microbial community, changing richness, diversity, and function of these communities (Torres *et al.* 2021).

One of the key functional traits that ensures *Acacia* spp. invasive success is their ability to grow in poor soils. This occurs due to rhizobia that live in root nodules, and perform biological nitrogen fixation (BNF), a process that converts gaseous atmospheric N into ammonia by the action of an enzyme (nitrogenase) (Dupont *et al.* 2012). In this symbiosis the bacteria benefit from a protective environment and receive dicarboxylic acids as a source of carbon and the plant receives ammonium from the rhizobia (Brockwell *et al.*2005). The process of BNF and the impact *Acacia* spp. causes on invaded habitats has been previously addressed, including the soil and belowground microbial communities under field conditions (Marchante *et al.* 2008; Souza-Alonzo *et al.* 2014).

The role of microbial communities in plant invasions has been studied recently, acknowledging that soil microbes might act as drivers of plant invasions, based on achievements in the above ground (Dawson and Schrama 2016). In *Acacia*, several studies performed on the nodulating bacteria showed that different genera are involved, mostly rhizobia, *Rhizobium*, *Bradyrhizobium Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Allorhizobium* as well as *Ochrobactrum* and *Ensifer* (Brockwell *et al.* 2005; Rodríguez-Echeverría *et al.* 2011; Souza-Alonso *et al.* 2017) and the same species (eventually the same tree) is able to form nodules with bacteria of several taxonomic groups (Brockwell *et al.* 2005). The origin of these symbionts is still under debate; while some studies indicate that *Acacia* spp. establish symbiotic relationships with co- introduced microbes, others suggest that new mutualisms are established in the invaded locations (Rodríguez-Echeverría *et al.* 2011; Souza-Alonso *et al.* 2017). Thus, biological fixation of nitrogen is one of the most important functional traits of this group of woody legumes, as is the ability to nodulate profusely (Rascher *et al.* 2011b, 2012).

The identification of the original source of N elucidates to what extent fixation is an important contribution to the N pool in plant cells. It has been based in the isotopic composition of a pool of N, allowing identification of the relative importance of sources that are isotopically distinct (Boddey *et al.* 2000). Stable isotopes are tracers of ecological processes, resulting from

the interaction between plants and environment. The $\delta^{15}N$ ($^{15}N/^{14}N$ ratio) signatures of certain plant tissues can be used for determining the source of N and quantifying the proportion of N derived from BNF. Since atmospheric N is used as the reference value for $^{15}N/^{14}N$ ratio, and soil processes generally discriminate against ^{15}N, near zero values in plant tissue are considered indicative of N derived by atmospheric N fixation (Dawson *et al.* 2002; Rascher *et al.* 2012; Ulm *et al.* 2017a, b). Another useful tracer of ecological processes and photosynthetic strategies is $\delta^{13}C$ signature, which has been used as a proxy for water use efficiency (WUE), in a large variety of both cultivars and native vegetation (Farquhar *et al.* 1989; Dawson *et al.* 2002). It reflects the ratio of CO_2 acquired to transpiration rate, via stomatal conductance; and can also be considered as an integrator of growth and phenological development (e.g., Werner and Máguas 2010).

Considering no studies have been performed on early *A. longifolia* development and its importance on outcompeting other species, underlying this species invasive ability, the present study intended to evaluate the combined effect of soil origins (forest, agricultural and dune), watering and nutrition regimes on juvenile *A. longifolia* development (1), and on symbiotic bacteria diversity levels (2). The habitats chosen included forest and agricultural soils, where *A. longifolia* is more invasive, as well as dunes in coastal areas, the habitat of its first introduction. We wanted to establish the key factors driving major responses at above and belowground levels, under controlled conditions in a greenhouse experiment. Acknowledging that soil is a living entity and that interactions between soil abiotic and biotic conditions have an impact of plant growth, we also included a preliminary approach on the symbiotic bacterial communities in the nodules from plants grown in the different soils and conditions.

Materials and Methods

Experimental design

Mature pods of *Acacia longifolia* were collected in Vila Nova de Milfontes (Odemira, Portugal), from trees growing in agricultural (37°68'26.38"N, 8°76'23.86"W), forest

(37°41'0.82"N, 8°46'11.40"W) and dune soil (37°41'30.53"N, 8°47'20.36"W). After collection, the seeds were manually removed from the pods, pooled into a single lot, and stored at room temperature (20-24 ºC). Prior to germination, seeds were surface sterilized with pure commercial bleach (containing sodium hypochlorite) for 5 min, rinsed with distilled water and transferred to a water bath at 100 ºC for 1 min. Seeds were germinated in Petri dishes containing filter paper moistened with water, incubated for 48h in darkness followed by a 16h light photoperiod for two weeks. The growing seedlings were individually planted into plastic dark pots containing approximately 1 L of one of the three soils [forest (F), agricultural (A) and dune (D)] and transferred to the greenhouse.

Following a two-weeks acclimation period, the shoot length was measured, and the experimental period was initiated. The combination of the three soils (F, A and D), with the two irrigation levels [high water availability (W+) *vs* low water availability (W-)] and the two nutrition levels [presence (N+) *vs* absence (N-) of nutritive solution, see below], resulted in a total of 3×2×2 = 12 different combinations, with 10 replicates each in a randomized design, which was fully re-randomized every week (Fig. 1). Hereon, for simplicity, these combinations will be referred to as "treatments" although they are simply the combination of the three factors of the experimental design and not treatments as used in the traditional sense in analysis of experimental designs. The irrigation regimes differed in the amount of water added, which was determined according to field capacity. The calculation of the field capacity was based on 5 measurements (independent measures, results not shown) for each soil tested, and the mean value obtained was used throughout the experiment, for all soil types and acacias developmental stage. The plants in the high-water availability regime were maintained at 70% of field capacity, corresponding to 35 mL of tap water (or nutrient solution, depending on the nutrition level). In regimes of low-water availability, irrigation was reduced to 30% of the field capacity, corresponding to 15 mL. Regardless of the treatment all plants were watered twice a week. Nutrient solution composition was based on the Hoagland solution (Hoagland 1933). Plants were grown in the greenhouse under natural photoperiod for 20 weeks, from November 2017 to April 2018. The greenhouse temperature was maintained between 18 ºC and 25 ºC.

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194 *Soil collection and analysis*

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196 Soil samples were collected in the vicinity of the acacias, up to a 1.5 m radius of the tree
197 or shrub, in Vila Nova de Mil Fontes, Portugal, in the same sites where seeds were collected. Soils
198 were collected at 20-30 cm depth. Dune soil was collected from a sandy area on a secondary dune,
199 with adult acacias growing. Forest soil was collected in a 50-years'old *Eucalyptus globulus* stand,
200 established before 1970's, in an area invaded by acacias for several years. Agricultural soils were
201 sandy soils with an intensive agriculture (alternating sweet corn and triticale, in August and
202 November, respectively) with fertigation, and herbicides for weed control. Acacias were also
203 growing next to this agricultural field. Soils of each type were collected from an area
204 representative of the site. All soils were manually homogenized into a composite sample using a
205 40 mm sieve. Three subsamples from each soil origin (forest, agricultural and dune) were
206 analysed at Laboratório de Análises de Solos e Plantas, UTAD – Universidade de Trás-os-Montes
207 e Alto Douro. Soils in which *Acacia longifolia* seedlings were grown were characterized by
208 texture, pH, organic matter percentage, electric conductivity, amount of total and mineral nitrogen
209 (NH_4^+ and NO_3^-), phosphorus (P), potassium (K), sodium (Na), calcium (Ca) and magnesium
210 (Mg).

211

212 *Above ground physiological measurements*

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214 Following the 20-weeks growth period, eight out of the ten replicate plants of each
215 treatment were removed from the pots. In each individual plant a set of parameters was measured:
216 (1) shoot length; (2) number of phyllodes; (3) phyllodes' fresh weight; (4) total leaf area.
217 Phyllodes were then stored in paper bags and oven dried at 60 ºC for at least 48h for dry weight
218 measurement and isotopic analyses (see below). A LI-3100C Area Meter was used to obtain total
219 phyllode area (4) while shoot length (1) was manually recorded with a ruler before phyllodes were
220 counted and removed from the plant so that phyllode fresh weight (3) could be measured with

scales. Based on these measurements, shoot increment and phyllodes water content was calculated with the following equations, respectively: (1) Shoot Increment = Final Length – Initial Length; and (2) Phyllode Water Content = [(Phyllode Fresh Weight – Phyllode Dry Weight) / Phyllode Fresh Weight] × 100.

Belowground physiological measurements

Excess soil was removed from the roots and the following measurements were performed for each plant: (1) root length; (2) number of nodules; (3) roots' fresh weight, evaluated after the removal of the nodules; (4) total nodule fresh weight. Root length measurements were recorded manually with a scale. Nodules of each plant were counted, manually removed from the roots, and roots and nodules were weighed separately. Roots were stored in paper bags and oven dried at 60 ºC for at least 48h for dry weight measurements.

Isotopic and pigments content analyses

Isotopic analysis was performed, using an average of two phyllodes and five nodules of three plants from each treatment. The samples were oven dried at 60 ºC for 48h. Following mechanical maceration with a ball mill (Retsch, Haan, Germany), 5 mg of phyllodes and 1 mg of nodules of the resulting fine powder were encapsulated into tin capsules. The samples were then analysed for $\delta^{15}N$, $\delta^{13}C$, nitrogen (%N) and carbon (%C) content at LIE-SIIAF (Stable Isotopes and Instrumental Analysis Facility, Faculty of Sciences, Lisbon University).

Additionally, photosynthetic pigments' content was evaluated. Three 0.5 cm diameter discs were removed from a phyllode in five of the eight replicates, with the aid of a manual punch. The discs were then suspended in 2 mL of methanol and stored at 4 °C in the dark, for 48h, after which absorbances were measured at 470 nm, 652.4 nm, and 665.2 nm with a Thermo Heλios β spectrophotometer (Thermo Electron Corporation). Chlorophyll *a*, *b*, total chlorophylls, and total carotenoids concentrations (in μg/mL) were calculated according to Lichtenthaler (1987).

Bacterial isolation, DNA extraction and fingerprinting

Nodules were surface disinfected in 1 min 70% ethanol followed by 6 min immersion in 5% sodium hypochlorite and rinsed in sterile distilled water. Disinfection success was confirmed by absence of growth following the imprinting of the nodules in fresh plates of YMA (Yeast Mannitol Agar) medium, incubated at 28 °C for 72h. The nodules were macerated using a sterile mortar and pestle and resuspended in 300 µL NaCl 0.85%, ensuring that the isolated organisms were from the inside the nodule. Of those, 250 µL were inoculated in YMA medium with 0.02 gL^{-1} of cycloheximide to prevent fungal contaminations. The Petri dishes were incubated at 28 °C, and growth was observed after 48 to 72 hours. Culture media used for bacterial isolation was previously sterilized by autoclaving at 121 °C for 20 minutes.

The resulting single colonies were restreaked three times for purification. The colonies were observed to discern shape and overall appearance, followed by Gram staining for characterization and differentiation of the isolates according to bacteria type and verification of colony purity. Oxidase test, KOH and catalase reactions allowed further characterization (Madigan *et al.* 2017).

DNA extraction was carried out based on a routine procedure for cell lysis, the boiling method (see Dimitrakopoulou *et al.* 2020): one to three colonies from each isolate were removed with a loop and suspended in 100 µL of TE buffer with 0.1 µL of Tween-20 and incubated for 10 to 12 minutes in a dry bath at 100°C, until lysis had occurred. The amplification of the samples was performed according to the Rep-PCR (Repetitive element sequence-based PCR) fingerprinting technique using PH and GTG5 primers. All amplification reactions were performed in a total volume of 25 µL, containing 1x PCR buffer, 3 mM of MgCl2, 0.2 mM of each dNTPs, 25 pmol of the primer, 1 U of Taq DNA polymerase (Invitrogen) and 2 µL bacterial lysate containing DNA. Each reaction included a negative control with all components except DNA. The amplification was performed with the BioRad 100 thermocycler. The PCR cycle used

consisted of an initial denaturation step of 5 min at 95 ºC, followed by 40 cycles of 1 min at 95 ºC, 2 min at 50 ºC, 2 min at 72 ºC and a final extension of 5 min at 72 ºC.

Amplification products were subsequently run in 1.2% (w/v) agarose gel electrophoresis at 90 V for 3 hours with 0.5x TBE as the gel and running buffer. The gels were then stained with 0.5 μgmL^{-1} ethidium bromide solution for 15 minutes and visualized through UV transilluminator Alliance 4.7 (Uvitec, Cambridge). After DNA migration, band profiles obtained in the agarose gel were compared using BioNumerics.

Statistical analysis

The statistical comparison of soils was done by performing a one-way ANOVA followed by a post-hoc analysis using Tukey multiple comparison test. Considering all the data, the first statistical approach was to verify the normality and homocedasticity of the sample population used for the experiment. The modified robust Brown-Forsythe Levene-type test showed that the hypothesis of homoscedasticity of the distribution of initial growth in plants in the 3 types of soils was not rejected (p-value = 0.9852). Normality assumption was also verified by the graphical distribution of data. On what concerns homogeneity, the Kruskal Wallis test showed an indistinct initial plant growth, with no evidence of effect soil type (p-value = 0.4671).

To explore the influence of the different treatments, the variables measured after the experimental period were grouped in two, representing the aboveground and belowground development of the juvenile plants. The variables selected to characterize the aboveground development were shoot increment, number of phyllodes, leaf area and phyllode water content. The variables chosen to characterize the belowground development were root length, number of nodules, total nodule weight and roots dry weight. Principal Component Analysis (PCA) was carried out separately for the sets of aboveground and belowground data, based on all plant individual measurements. The motivation behind this approach was to reduce the complexity whilst still integrating information from the complete set of measurements. The scores of the first principal components, derived from each set of variables (aboveground development and

belowground development) were used as proxy for the above ground and belowground plant development. Here-on, we will refer to these new variables as the shoot vigour and the root development. Choosing the first principal component (PC1), assured that the new variables built to quantify plant development accounted for the largest possible variance in the data. Principal components were obtained considering the eigenvectors of the correlation matrix of the original data, which is equivalent to the standard procedure in PCA that consists in standardising the data prior to computing the principal components. To make most use of the available data, a nearest neighbour imputation algorithm was used to complete the data of the variable roots dry weight. The missing values were imputed as the median of the 3-NN (Nearest Neighbours). Nearest neighbours were identified using Gower distance to avoid bias due to scale differences between the variables. The correlation matrix of the imputed data set did not differ from the correlation matrix of the original data, assuring that the principal components found were not influenced by imputation.

A three-way ANOVA was conducted to assess the main and interaction effects of the combination of the abiotic factors in study (soil, watering, and nutrition) on the above and belowground plant performance, based on the individual projections on the first PCA axis concerning aboveground and belowground development. Data regarding photosynthetic pigments and stable isotopes were also analysed using a three-way ANOVA, with soil, watering, and nutrition as the main factors. Comparison between treatments was done through Tukey multiple comparisons test using function *TukeyHSD*. All statistic were performed with R and RStudio (version 3.6.1, R Core Team 2016), with p-values adjusted for multiple testing. Additionally, correlation between shoot vigour and root development variables was calculated to assess the association between the two under each of the factors in study (soil, watering, and nutrition).

Bacterial PCR fingerprinting profiles were compared using BioNumerics software (Applied Maths, Sint-Martens-Latern, Belgium). Cluster analysis and resulting dendrogram were performed using the Pearson correlation coefficient as association measure and the unweighted pair-group method with arithmetic mean algorithm (UPGMA) as the clustering algorithm. Shannon–Wiener and Simpson diversity indexes were used to calculate the diversity and evenness

of the bacterial isolates (Krebs 1989). Data from the dendrogram was converted into a radar plot to get a clearer picture of bacterial richness and similarity according to soil type and treatment combination.

Results

Soil chemical properties

Soils in which *Acacia longifolia* seedlings were grown had distinct chemical properties which can briefly be resumed as forest soil having a higher organic matter content and being more acidic, while agricultural soil had the lowest organic matter but an overall higher nutrient availability (Table 1). Dune soils had an intermediate composition. The comparison of forest and agriculture soils revealed they were similar in terms of texture, NH_4^+ and sodium (Na) availability but showed distinct chemical composition in several of the other parameters analysed. Statistically significant differences were found between agricultural and forest soil regarding organic matter content, pH, electric conductivity, mineral nitrogen (NO_3^-), phosphorus (P), potassium (K) calcium (Ca) and magnesium (Mg). Forest soil was more acidic (pH = 4.8) and presented lower values in the mentioned parameters compared to agricultural soil (Table 1). Statistically significant differences were found between dune and forest soil, regarding organic matter content dropping to half in dune soils, and pH, which is higher in dunes. The comparison of soil composition between agricultural soils and dunes were all non-significant.

Descriptive analysis of plant growth under different treatments and functional evaluation

Graphical analysis suggests that acacias' early growth is not influenced only by the soil origin, but also by the different combinations of watering and nutrition (or treatments, see Fig. 1 for experimental design). Shoot increment and number of phyllodes, indicative of a higher plant growth, were recorded in agricultural (A) and forest (F) soils (Fig. 2a, b). Dune (D) plants

presented a similar growth under high water availability (W+) in combination with the presence of nutrient (N+) solution (Fig. 2a, b; Supplementary Information, Table S1). This higher aboveground growth was accompanied by higher root length and number of nodules (Fig. 2d, e). However, in the absence of nutrient solution but high-water availability (W+N-), shoot and root growth, number of phyllodes and root nodules decreased (Supplementary Information, Table S1). Phyllodes' water content was lower for plants in dune soils (Fig. 2c), while total nodule weight had similar values regardless of the type of soil, watering, and nutrition treatment, although mean values tended to be lower in treatments with low water availability (Fig. 2c, f).

Considering the photosynthetic pigment analysis performed at the tip and base of the phyllodes, carotenoids content (Fig 2g, h) was higher under low-water availability with added nutrition (W-N+), a pattern observed for dune soils. Other photosynthetic pigments displayed similar tendency (Supplementary Information, Table S1).

The highest isotopic $\delta^{15}N$ values were found in phyllodes from plants grown in agricultural soil, either with high-water availability (W+) or low-water availability (W-) (Fig. 2i; Supplementary Information, Table S2). Both soil type and nutrition have effect on $\delta^{15}N$ values, and in the treatment AW+N-, as well as in combinations of forest soil and absence of nutrient solution (FW+N- and FW-N-), $\delta^{15}N$ values were higher than those obtained from plants growing in dune soil. Despite differences in the isotopic N values, the phyllodes had 3% nitrogen content regardless of treatment. Phyllode $\delta^{13}C$ content showed insignificant differences between the treatments, varying from -31.3 to -32.6‰ (Supplementary Information, Table S2).

Principal component analysis for the plant growth parameters

Shoot vigour (corresponding to the first principal component for the aboveground development features) accounted for 63% of the variation in the data (Supplementary Information, Fig. S1). Shoot increment, number of phyllodes and leaf area have positive weights in the first principal component whereas phyllodes water content has a negative weight. The three-way ANOVA revealed a significant influence of soil origin ($p \leq 0.001$), nutrition ($p \leq 0.001$) and a

388 combined effect of watering and nutrition ($p \leq 0.01$) on the shoot vigour of the plants (Table 2).
389 Despite the absence of statistical significance, there was an underlying combined effect of all
390 three abiotic factors (soil, watering, and nutrition; Table 2). Overall, greater shoot vigour was
391 found in forest and agricultural soil, with high water availability (W+) and the presence of nutrient
392 (N+) solution (Fig.3, FW+N+ and AW+N+). Absence of nutrition combined with high water
393 availability is associated with a lower shoot vigour in plants grown in the three soil types, as well
394 as in DW-N-, with significant differences with the FW+N+ (Fig. 3; Supplementary Information,
395 Table S3). Shoot vigour in plants growing in agricultural soils was not different despite varying
396 the water or nutrition treatments, however in plants grown in forest soils with high water
397 availability and in the absence of nutrition, shoot vigour decreased. Plants grown in DW+N- were
398 the ones presenting the lower shoot vigour values. DW+N- was found to be different from
399 FW+N+, AW+N+, AW-N-, AW-N+, DW-N+ and FW-N-. In Fig.3, mean shoot vigour and
400 corresponding 95% confidence intervals are plotted allowing a graphical comparison of all the
401 treatments. This information is complemented with Table S3 (Supplementary Information), where
402 significant mean differences between pairs of treatments, along with the corresponding
403 confidence intervals and adjusted p-values, can be found.

404 Root development (corresponding to the first principal component for the belowground
405 development features) accounted for 49% of the variation in the data (Supplementary Information,
406 Fig. S2). All features (root length, number of nodules, total nodule weight and roots dry weight)
407 had a positive weight in the formulation of root development. The analysis of variance found a
408 significant effect of soil ($p \leq 0.01$), nutrition ($p \leq 0.001$) and their combination ($p \leq 0.05$), as well
409 as the combination of watering and nutrition ($p \leq 0.01$; Table 2). In general, there was a greater
410 root development of plants grown in forest soil, particularly under high-water availability and
411 presence of nutritive solution (Fig. 3, FW+N+). A similar root development was obtained in plants
412 from dune soils in the same conditions (DW+N+), however roots showed lower development in
413 dune soils in the absence of nutrient solution (DW+N-, Supplementary Material, Table S3). Root
414 development in FW+N+ differed significantly from DW+N-, AW-N+, DW-N-, AW+N+,

AW+N- (Fig.3). In agricultural soil, root development showed little variation, regardless of treatment combination.

When assessing overall plant development, a significant positive correlation (r = 0.54, 95% CI = 0.376 - 0.665) was observed between shoot vigour and root development considering all treatments.

Nodule bacterial diversity analysis

A total of 150 colonies were initially obtained from root nodules collected in plants grown under different soils: 40 colonies from forest soil, 61 from agricultural soil and 49 from dune soil. However, some bacteria were lost in the purification process, due to the absence of growth after being restreaked. After purification, a total of 111 isolates were obtained, 35, 45 and 31 from forest, agricultural and dune soil, respectively.

Bacterial community present in the nodules was fingerprinted considering the applied treatments, including soil type. The dendrogram obtained (Supplementary Information, Fig. S3) combines the PCR fingerprinting results from both PH and GTG5 amplification, as well Gram staining and biochemical tests. Cluster analysis indicated that bacterial isolates grouped in four main clusters, with varying degrees of similarity. To further understand the bacterial community obtained from root nodules, Shannon-Wiener and Simpson indexes were calculated. Considering a cut-off of 50% and a total of 51 groups and 111 strains, a Simpson index (D) of 0.9768 and a Shannon-Winer index score (H') 0.1399 were obtained. Although no clear association between the different treatments and the microbial community diversity in the dendrogram, a radar plot analysis showed that cluster Ia groups bacterial isolated from all treatments, but mostly from agricultural soils (Fig. 4). Furthermore, bacteria isolated from nodules from agricultural soils revealed a lower diversity and were all grouped in cluster Ia and Ib. No isolates were clustered in the two other main groups, Ic and II, which included only isolates obtained from plants growing in dunes or forest soils.

Discussion

How does agroforest and sand dune soils impact the development of Acacia seedlings?

Our study aimed to address *Acacia longifolia* juvenile plant development under different soils, watering, and nutrition conditions, to establish the influence of these factors on the interactions that contribute to major above-below responses of this invasive species. Indeed, the expansion of this exotic invasive species in different agroforestry as well as in natural dune systems, leads to several questions related with the early phase development of plants under these conditions. Early plant development will have vital impact on invasive behaviour and success, so there is a need to evaluate the role of soil, nutrition, and water on early *A. longifolia* growth.

In the present study, the composition of agricultural, forest and sand dunes soils showed differences, with forest soil being more acidic and with a higher organic matter content. This observation is probably related with a heavily invaded location, with litter and foliage recycling (Rodríguez-Echeverría *et al.* 2009; Souza-Alonso *et al.* 2014). Forest and agricultural soils were the more dissimilar, considering both organic matter and nutrient availability, while soils in sand dunes had an intermediate composition. Dune soil was associated with secondary dunes, with adult acacias growing and consequent foliage natural recycling. No major differences were found between dunes and agricultural soils, since the latter were also sandy soils, and although being subjected to intensive agriculture practices, some leaching probably occurred. However, soils itself cannot explain most of the differences found in acacia's development, suggesting several interactions are taking place between abiotic and biotic factors, which are not easy to interpret. As reviewed by Igiehon and Babalola (2018), soil properties can select specific microorganisms by creating conducive environments favouring certain taxa. Considering the invasive capacity of *Acacia* species, it is important to highlight the pivotal role of the above–belowground components in the invasion strategy, including the interactions between plants and soil microorganisms (Vestergärd *et al.* 2015).

Seedlings of acacias growing in dune soils with absence of nutrition had a lower development, both above and belowground, resulting from low organic matter and nutrients content, in particular phosphorus. Dunes are a poor soil, mainly arenosols, with a lower capacity for water retention and, consequently, higher nutrient loss. Above and belowground indicators of lack of nutrients were the lower phyllode number and leaf area, as well as nodule weight (Fig. 2). These characteristics have also been associated with phosphorus deficiency (Leidi and Rodríguez-Navarro 2000; Divito and Sadras 2014), however its effects are neither completely understood nor consensual (Divito and Sadras 2014). In dune soils in the absence of nutrition there is a major drop in nodule number, suggesting that lack of nutrition may be more limiting than lack of water in the experimental conditions (see below). There was a combined influence of watering and nutrition, particularly on belowground development since plant nitrogen and phosphorus uptake are highly dependent on water availability in the soil (Mariotte *et al.* 2020). Reduced biomass in plants growing in dune soil has been previously reported for *A. longifolia* young plants in comparison to other soil types (Rodríguez-Echeverría *et al.* 2009). Another interesting observation of the present study is a similar root/shoot ratio for acacias growing in different soil (see Supplementary Information, Table S1), as reported previously (Rodríguez-Echeverría *et al.* 2009), revealing this fixed parameter for this species. However, plants grown in forest and dune soils revealed a better equilibrium in the above/belowground development (Fig. 3) and showed the largest and smallest size of all conditions tested. Plants grown in dune soils in high-water availability and absence of nutrition (DW+N-) had the lowest root development, whereas the lowest shoot vigour was found in dunes with low water availability and absence of nutrition (DW-N-).

Acacias grown in agricultural soil presented little variation among treatments in the aboveground component, showing that plants are more resistant to “disturbance” (watering or nutrient limitation) in this managed soil (Fig. 3). In agricultural soils, the presence of higher nitrate and phosphate in the soils (Table 1) allowed for shoot vigour and the absence of nutrition didn’t impact on growth. Photosynthetic pigment concentrations in plants grown in agricultural soil were slightly lower, maybe due to larger phyllode size (Supplementary Information, Table S1).

Surprisingly, forest soils with high-water availability and presence of nutrition (FW+N+) provided the best conditions for growth, as shown by shoot and root development (Fig. 3). However, in the absence of nutrition (FW+N-), forest soils can also limit acacias' above ground development where growth was comparable to that obtained in dune soils, for the different watering and nutrition regimes. For the belowground component in forest soils, the same effect of nutrition in limiting growth in acacias could be verified (higher root development in FW+N+ in comparison with FW+N-).

Recent findings indicate the role of soil abiotic factors as a conditioning factor, especially in the early stages of invasion (Meira-Neto *et al.* 2018; Vestergärd *et al.* 2015). Acacias notoriously alter soil properties in the invaded sites, creating favourable conditions which potentiate their development (Ulm *et al.* 2017a, b) and the establishment of symbiotic relationships with soil bacteria. However, they must overcome the soils' limiting influence, particularly when soils are poor in nutrients (Le Roux *et al.* 2018). Birnbaum *et al.* (2014), reported no effect of soil on plant development, including *A. longifolia*, but one important difference must be pointed out: in the mentioned study, the soil of origin did not compose the entirety of the plants' growth substrate, while in the present study the acacias were grown in soil originally collected from the different locations.

Nutrition versus *atmospheric nitrogen fixation*

Acacias grown in forest and dune soil in the presence of nutrition (N+) had a higher belowground development, but in the absence of nutrition dune pants showed a decrease in development and results are indicative of a lower number of nodules. A lower belowground development was also observed in juvenile acacias grown in agricultural soil, particularly in the presence of nutrient solution, where plants formed fewer nodules and registered a lower nitrogen fixation. This inhibitory effect of N (nitrogen) on nodule formation (number of nodules) and growth of root nodules (weight of nodules) has been reported previously for other leguminous plants (Xia *et al.* 2017). Nodulation is a high energy demanding process, and in environments

where there is a high nutrient availability, it occurs less or is even unnecessary (Stephens and Neyra 1983). Moreover, it has been reported that the presence of a high nitrate content led to nodule senescence, or inhibited its formation, eventually downregulating nitrogen fixation (Dupont *et al.* 2012). These may be some conditioning factors that explain the low number of nodules in plants grown in agricultural soil, contributing to its belowground development being lower compared to the aboveground. Plants grown in the nutrient-rich agricultural soil revealed only minor changes in response to treatment combinations in the above and belowground components, except in the number of nodules and phyllode $\delta^{15}N$.

Given the importance of atmospheric nitrogen fixation by the nodules, an evaluation of such symbiotic N fixation and the correspondent BNF contribution to the N total uptake was assessed trough standard phyllode $\delta^{15}N$ signature. As largely described in the literature for different leguminous plants, including *A. longifolia*, *Stauracanthus spectabilis*, *Ulex europaeus* and *Cytisus grandiflorus* (Rodríguez-Echeverría *et al.* 2009; Hellmann *et al.* 2011; Rascher *et al.* 2012; Ulm *et al.* 2017a, b), $\delta^{15}N$ values near 0‰ indicate atmospheric N fixation. In the present study, in the absence of nutrition, active processes of BNF were occurring, suggested by phyllode $\delta^{15}N$ isotopic analysis, with values in accordance with previously reported data for other nodulating legumes (Boddey *et al.* 2000). Depletion of phyllode $\delta^{15}N$ values was much more pronounced in acacias from dune soil, which registered average signatures close to the atmospheric value of 0‰, in the treatments DW+N- (-0.13‰) and DW-N- (-1.5‰) (Fig 2i; Supplementary Information, Table S2). Phyllode nitrogen content was similar in acacias from all three soils, suggesting that plants grown in dune soil were able to surpass the N limitation. Furthermore, on what concerns the nodules isotopic N analysis, they always showed a positive and less variable $\delta^{15}N$ for all plants, regardless of growing conditions.

Finally, watering regime *per se* had a minor effect in plant development, both in the above and belowground components, as revealed by the leaf $\delta^{13}C$ content remaining unaffected by changes in watering. Previous studies reported the importance of water in *A. longifolia*, with overall plant development hindered when seedlings were submitted to water stress (Morais and Freitas 2012). However, in the present study, experiments were performed in the greenhouse

554 during winter, and temperature was never above 25 °C, suggesting that water stress conditions
555 were not extreme. Nevertheless, water treatment could not be removed from our experimental
556 design due to interaction with other factors, such as nutrition.
557
558 *Is there a difference in bacteria diversity in the different soils?*
559
560 In the present study, we performed a preliminary approach on the symbiotic bacterial
561 communities in the nodules from plants grown in the different soils and conditions to evaluate
562 diversity levels. Overall bacterial diversity assessed through cultivable bacteria was high,
563 corresponding to over one hundred isolates obtained from *A. longifolia* root nodules, reinforcing
564 acacia's status as a generalist species (Birnbaum *et al.* 2014; Rodríguez-Echeverría *et al.* 2011).
565 We envisage that part of the bacterial diversity was lost, since more obligate symbionts were
566 either unable to grow *in vitro* or lost in the cultivation process. Even though no species
567 identification was performed in our study, previous research indicate that acacias are mainly
568 nodulated by bacteria belonging to the genera *Bradrhizobium,* particularly *B. japonicum*,
569 *Rhizobium*, *Mesorhizobium* and *Sinorhizobium,* as well as *Ensifer* and *Acinetobacter* (Rodríguez-
570 Echeverría *et al.* 2003; Fterich *et al.* 2012; De Meyer *et al.* 2015; Souza-Alonso *et al.* 2017;
571 Kamutando *et al.* 2017, 2019; Jesus *et al.* 2020). Although the bacterial community associated
572 with acacias can vary, the genus *Bradyrhizobium* seems to be shared across most locations and is
573 in association with several acacia species. The importance of this genus relates to the fact that it
574 is one of the main rhizobial taxa to express genes associated with plants promoting traits, such as
575 the metabolism of nutrients, vitamins, and nitrogen (Kamutando *et al.* 2017, 2019). Therefore,
576 the genus will play an essential role in plant development and in the processes underlying
577 invasiveness success. Recently our group has showed that despite *Bradyrhizobium* spp.
578 dominance in *A. longifolia*, *Paraburkholderia* spp. and *Pseudomonas* spp. are important bacteria
579 present in the nodules, and major partners in the symbiosis (Jesus *et al.* 2020).
580 Cluster analysis in the present study showed that bacteria obtained from agricultural soils
581 cluster into two groups (Supplementary Information, Fig. S3: Ia and Ib;), revealing a higher

582 genetic similarity than the one present in bacterial communities obtained from forest and dune
583 soils. This reflects the lower variability in the bacterial communities present in more managed
584 agricultural soils, where nitrogen addition causes a decline in bacterial species richness and
585 diversity and a shift of bacterial composition (Li *et al.* 2019). As suggested by Igiehon and
586 Babalola (2018), the level of chemical fertilizer application constitutes an important
587 anthropogenic factor modulating rhizosphere bacterial diversity. In comparison, natural
588 ecosystems are richer in bacterial variability that are available to colonize acacia's roots and form
589 nodules. Furthermore, acacias grown in agriculture soils presented a decrease in the number of
590 nodules and a higher $\delta^{15}N$, in the presence of nutrition, all suggesting a lower bacterial activity.
591 The number of nodules has been previously considered as a proxy for symbiotic success (Le Roux
592 *et al.* 2018), corroborating this decrease in microbial diversity. These differences in bacterial
593 diversity should be further exploited, to help understand the role of symbiotic bacterial
594 communities in plant performance and invasion success.
595
596 **Conclusions**
597
598 This study emphasizes the relevance of soil nutrition and biotic-abiotic interactions in the
599 overall development of *Acacia longifolia*, clarifying the causes of different expansion at dune and
600 agroforest soils. In the present study, maximum growth was obtained in plants grown in forest
601 soils with high water availability with addition of nutrition, while minimum growth was obtained
602 in soil dunes in the absence of nutrition. The main constraint in dune soils is nutrition, limiting
603 nodule number, while forest soils provide an environment where acacias are well adapted and can
604 disseminate. However, forest soils are also affected by nutrition and the limitation of nutrition
605 under high-water availability cause a decreased growth. Agricultural soils seemed to favour the
606 shoot vigour and constant management is required to prevent acacia's expansion. In the
607 agriculture anthropogenic artificial soil, plants invested in above ground growth, maintaining a
608 belowground development similar regardless of water and nutrient variation. Our study points to
609 an important effect of nutrition, which can be unique (promote or inhibit growth) depending on

soil type. Abiotic factors must then be considered in an integrative perspective since they act in concert to shape plant development. Biotic factors also play an important role, and our study shows that bacteria diversity was shaped mainly by soil properties. A higher diversity was found in bacterial communities in nodules from forest and dune soils, providing a major advantage for acacia's invasion in those habitats. Therefore, development and expansion of *A. longifolia* is based on soil conditions, and the microbial partners that will be able to take advantage of the abiotic factors and thrive, expanding their growth below and aboveground, increasing the invasibility of this species. These conditions are more common to find in forest and dune soils, when water is available, rendering acacias a threat to native species and habitats.

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Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Data availability statement

Further data and details are provided in the Supplementary Information file. The full dataset used in this study is available upon request to the corresponding Author.

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Tables

Table 1: Physical and chemical characterization of the three different soil origins according to texture, pH in water, organic matter content, electric conductivity**,** and nutrient availability. Mean values are presented. Statistically significant differences, according to Tukey post-hoc test after one-way ANOVA with $\alpha = 0.05$ are represented by different letters.

Type of Soil	Texture	pH water	Organic matter (%)	Electric conductivity (1:5) dS m^{-1}	Extraction in water (1:5) - mg element kg^{-1}						
					NO_3^-	NH_4^+	P	K	Na	Ca	Mg
Forest	coarse	4.77^a	2.22^a	0.05^a	1.82^a	1.36^a	0.91^a	23.8^a	6.33^a	92.47^a	66.13^a
Agricultural	coarse	6.03^b	0.39^b	0.26^b	68.53^b	0.51^a	11.77^b	136.7^b	72.93^a	818.7^b	427.7^b
Dune	coarse	6.03^b	0.84^b	0.18^{ab}	43.97^{ab}	1.42^a	4.45^{ab}	83.60^{ab}	70.83^a	539.0^{ab}	274.3^{ab}

879 **Table 2:** Three-way ANOVA summary of the effects of using soil, watering and nutrition, and
880 their interactions on the variables shoot vigour and root development. Bold values highlight
881 significant effects.

	Shoot vigour					Root development				
	Df	Sum of squares	Mean square	F value	Significance	Df	Sum of squares	Mean square	F value	Significance
Soil	2	5581	2791	8.352	**≥ 0.001**	2	2585	1292.6	5.682	**0.004**
Watering	1	424	424	1.268	0.263	1	218	218.5	0.960	0.329
Nutrition	1	7603	7603	22.753	**≥ 0.001**	1	2074	2074	9.117	**0.003**
Soil:Watering	2	707	353	1.058	0.352	2	163	81.4	0.358	0.700
Soil:Nutrition	2	848	424	1.268	0.287	2	1944	972.2	4.274	**0.017**
Watering:Nutrition	1	3778	3778	11.308	**0.001**	1	1170	1169.7	5.142	**0.026**
Soil:Watering:Nutrition	2	1822	911	2.727	0.071	2	325	162.5	0.714	0.429
Residuals	84	193.304	28069			84	19109	227.5		

882

883 **Figure captions**

884 **Fig. 1:** Description of the experimental design and treatment combinations (3x2x2) applied to
885 seedlings of *A. longifolia* (n = 10) during the 20-week experimental period

886 **Fig. 2:** Boxplots showing data distribution by soil type and treatment of shoot increment (a),
887 number of phyllodes (b), phyllode water content (c), root length (d), number of nodules (e), total
888 nodule weight (f), carotenoids content in phyllode tip (g), carotenoids content in phyllode base
889 (h), and $\delta^{15}N$ phyllode signatures (i) in plants of *A. longifolia*, measured after a growth period of
890 20 weeks in three different types of soil. A – agricultural soil; D – dune soil; F – forest soil; W-
891 N- – water at 30% field capacity/absence of nutrient solution; W-N+ – water at 30% field
892 capacity/presence of nutrient solution; W+N- – water at 70% field capacity/absence of nutrient
893 solution; W+N+ – water at 70% field capacity/presence of nutrient solution.

894 **Fig. 3:** Forest plot for the effect of the different treatment combinations on variables shoot vigour
895 (a) and root development (b), obtained through three-way ANOVA. The square and lines
896 represent the mean and 95% confidence interval, respectively, of the variable in each treatment.
897 Statistically significant differences (not family wise adjusted) between treatment combinations
898 are found when the correspondent lines of the confidence intervals do not overlap. Mean
899 differences and Tukey multiple comparisons of means adjusted p-values and correspondent 95%
900 family-wise confidence intervals are presented in Supplementary Information, Table S3. A –
901 agricultural soil; D – dune soil; F – forest soil; W+N+ – water at 70% field capacity/presence of
902 nutrient solution; W+N- – water at 70% field capacity/absence of nutrient solution; W-N+ – water
903 at 30% field capacity/presence of nutrient solution; W-N- – water at 30% field capacity/absence
904 of nutrient solution.

905 **Fig.4:** Radar plot characterising the treatments with respect to bacterial diversity. Each vertex in
906 the radial represents a treatment (combination of soil, watering, and nutrition conditions), the
907 equidistant concentric radar lines represent proportions 0%, 25%, 50% 75% and 100%, and the
908 coloured webs represent the clusters. In each radial line the allocation of the bacterial diversity

found in the treatment can be read. For example, (i) all bacteria found in AW-N- are in cluster Ia; (ii) bacteria found in DW+N- are equally distributed in clusters Ia, Ib and II, and less present in cluster Ic.