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Influence of abiotic factors on growth and nodulation of *Acacia longifolia*

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Resumo

O género Acacia está entre os invasores mais agressivos em todo o mundo. Este grupo de plantas leguminosas causa sérios problemas nos habitats em que é introduzido, uma vez que modifica drasticamente as comunidades nativas, provoca alterações no solo, nomeadamente nos reservatórios de carbono e azoto, e ainda no ciclo da água. Uma vantagem competitiva importante da maioria das acácias em relação às plantas nativas resulta da possibilidade de crescer e prosperar em solos muito pobres, por terem a capacidade de desenvolver nódulos fixadores de azoto, que promovem a sua nutrição e desenvolvimento. Portanto, a introdução de Acacia diminui a diversidade biológica e altera o funcionamento dos ecossistemas.

Em Portugal, uma das espécies mais problemática é a *Acacia longifolia*. Esta espécie foi introduzida no nosso país pelos serviços florestais como forma de preservar e fixar dunas de algumas zonas costeiras. Desde a sua introdução nos séculos XIX e XX, a espécie colonizou os ecossistemas agro-florestais e dunares, perturbando o seu funcionamento e alterando o equilíbrio nas comunidades de flora nativa. O potencial invasor e a capacidade de colonização desta espécie exótica deve-se, em parte, à sua capacidade de estabelecer relações simbióticas com bactérias fixadoras de azoto presentes no solo. A fixação de azoto ocorre dentro dos órgãos recém-formados nas raízes da planta, designados por nódulos, como resultado de uma simbiose estabelecida entre a planta e bactérias colectivamente designadas por rizóbio. Vários estudos têm sido realizados, relativamente ao processo de fixação biológica de azoto e no impacto que *Acacia* spp. tem sobre os habitats que invade, especialmente no solo e nas populações endémicas. No entanto, sabe-se pouco sobre a forma como os factores abióticos, por exemplo, as características do próprio solo, podem influenciar e condicionar o desenvolvimento das plantas.

Neste trabalho temos como objectivo compreender como as condições abióticas podem potenciar ou limitar o desenvolvimento de *A. longifolia*. Nesse sentido pretendemos comparar o desenvolvimento de acácias germinadas em tipos três solos – agrícola, florestal e dunar, submetidas a diferentes condições de nutrição e irrigação. Pretende-se assim compreender, e como estes factores vão condicionar a formação de nódulos radiculares, bem como o estabelecimento de relações de simbiose entre a planta e microorganismos fixadores de azoto presentes no solo.

Para tal, foram recolhidas amostras de sementes e solo da região de Vila Nova de Mil Fontes. As sementes foram germinadas e, posteriormente, desenvolvidas em estufa, em três tipos diferentes de solos - agrícola, florestal e dunar. Adicionalmente as plantas foram sujeitas à conjugação de quatro tratamentos – irrigação a 30% ou 70% da capacidade de campo, presença ou ausência de solução nutritiva. As plantas tiveram um período de crescimento de vinte semanas e ao fim desse período foram avaliadas, através da medição, em cada planta, dos seguintes parâmetros: (1) comprimento aéreo e radicular; (2) área total da folha; (3) número e peso fresco dos nódulos, filódios e raízes e (4) níveis relativos de clorofila, medidos com SPAD-502. Adicionalmente três discos de filódios foram removidos de algumas das plantas em estudo, não só de modo a avaliar o conteúdo absoluto de pigmentos fotossintéticos, como também numa tentativa de estabelecer uma curva de calibração com as leituras de SPAD. O processo de fixação de azoto foi avaliado tendo por base a composição isotópica de carbono e azoto, tanto nos nódulos, como nos filódios.

Adicionalmente, as comunidades bacterianas presentes nos nódulos radiculares foram isoladas. Primeiramente, os nódulos de cada solo e respectivo tratamento foram agrupados e desinfectados. Após confirmação da sua desinfecção por impressão em caixa de Petri, os mesmos foram macerados e suspendidos em NaCl 0.85% . Posteriormente, esta suspensão foi inoculada em meio de manitol e incubada durante 48 a 72 horas. Por repicagens sucessivas, foram obtidas colónias puras, procedendo-se à lise das células, através do *boiling method*, seguido da análise molecular por *fingerprinting*. A análise foi realizada através da técnica de Rep-PCR, tendo sido testados três *primers*

– M13, PH e GTG5. Os produtos obtidos por amplificação foram separados por electroforese em gel de agarose e as imagens resultantes foram capturadas com UV transiluminador Alliance 4.7 (Uvitec, Cambridge) e analisadas com o *software* BioNumerics, de forma a avaliar a diversidade bacteriana presente.

A análise de dados realizada após levantamento do ensaio, revelou que o desenvolvimento dos exemplares de *A. longifolia* foi principalmente afectado pelo solo e pelo regime nutritivo. As acácias desenvolveram-se mais em solo florestal e agrícola, traduzindo pelo maior crescimento da parte aérea e pelo maior número de filódios. No entanto, as plantas desenvolvidas neste último tipo solo, apresentaram um menor número de nódulos. Em contraste, no solo dunar registou-se um menor desenvolvimento, particularmente em termos de crescimento, número e área dos filódios. Estas diferenças deveram-se principalmente às características do solo, regime nutritivo e à sua interacção.

O solo agrícola foi um solo artificialmente alterado, sujeito a fertilização, com elevada disponibilidade nutritiva. O solo florestal teve uma elevada quantidade de matéria orgânica. O solo dunar apresentou uma composição intermédia, em termos de disponibilidade de matéria orgânica e de nutrientes. Logo, uma maior disponibilidade nutritiva levou por um lado a um maior desenvolvimento e por outro, a uma menor nodulação e fixação de azoto. De forma análoga, considerando o regime de solução nutritiva, os exemplares às quais esta foi adicionada tiveram um maior crescimento, resultante da maior disponibilidade de nutrientes, que tende a promover o desenvolvimento das plantas. Houve, tendencialmente, um maior incremento (aéreo e radicular) na presença de solução nutritiva e conforto hídrico (W+N+), particularmente nos solos florestal e agrícola. Em suma, a acção simultânea dos diferentes factores abióticos influenciou o desenvolvimento da planta. No entanto, a influência dos diferentes factores e conjugações de tratamentos foi diferente consoante o solo em que foram aplicados. As plantas que se desenvolveram em solo agrícola, por ser rico em nutrientes e com grande capacidade de retenção de água, foram de modo geral pouco afectadas por alterações nos tratamentos, enquanto que as que se desenvolveram em solo dunar (poroso e pobre em nutrientes), viram desenvolvimento condicionado pelos diferentes regimes de nutrição e irrigação. Os nossos resultados evidenciaram ainda que em situação de menor disponibilidade nutritiva houve uma maior fixação de azoto atmosférico, o que se traduziu em menor conteúdo $\delta^{15}N$. Esta tendência foi transversal aos vários tipos de solo, mas foi particularmente evidente no solo dunar, onde foram registados os valores mais baixos. Quanto às concentrações de pigmentos fotossintéticos, houve uma tendência para que fossem mais baixas em solo agrícola, resultantes de filódios com maior área e maior conteúdo hídrico.

Quanto à análise da diversidade bacteriana, foram obtidos 111 colónias a partir dos nódulos radiculares. O *fingerprinting* obtido com os *primers* PH e GTG5 evidenciou não só a diversidade e heterogeneidade bacteriana associada a *A. longifolia*, como também o caracter generalista da espécie. No entanto não foi possível estabelecer qualquer correlação entre as colónias obtidas e as combinações de tratamentos aplicadas durante o ensaio experimental, o que indica que estes tratamentos não influenciaram o número de bactérias em simbiose com *A. longifolia*. Não foi também possível correlacionar o desenvolvimento das acácias com a diversidade bacteriana obtida em cada solo e respectivo tratamento. Estes resultados são, no entanto, preliminares, pelo que será necessária a realização de mais estudos que permitam a identificação das bactérias, e deste modo melhor compreender se há uma correlação entre o desenvolvimento da planta e as estirpes bacterianas presentes nos nódulos radiculares.

Relativamente à calibração entre as leituras de SPAD e os níveis absolutos de pigmentos fotossintéticos, foram obtidos baixos coeficientes de ajustamento, entre 0.1 e 0.3, para os modelos calculados. Apesar destes valores, a relação entre as leituras espectrofotométricos e de SPAD-502, de

clorofila e de carotenóides, foram estatisticamente significativa. Logo, as equações de calibração obtidas possuem capacidade preditiva, sendo, no entanto, necessário a análise de mais amostras para validar o modelo.

Como conclusão deste trabalho, e considerando a *Acacia longifolia* uma espécie invasora extremamente agressiva que perturba e modifica habitats e comunidades tanto em Portugal como noutras regiões do mundo, o estudo dos factores que influenciam o desenvolvimento da espécie é essencial. Uma vez que o sucesso de *A. longifolia* resulta da conjugação e interacção de vários factores, serão necessários mais estudos que sigam uma perspectiva integrativa.

Com este trabalho, pretendeu-se dar-se uma contribuição para o melhor entendimento dos processos subjacentes ao sucesso de *A. longifolia* como invasora, tais como o papel dos factores abióticos na modulação do desenvolvimento da planta e a importância da fixação biológica de azoto, particularmente em situações de escassez de nutrientes, de forma a encontrar estratégias para evitar o seu estabelecimento e controlar a sua dispersão.

Palavras-chave: *Acacia longifolia*, factores abióticos, nódulos, fixação de azoto

Abstract

The genus Acacia is amongst the most aggressive invaders worldwide. This group of leguminous woody plants causes severe problems in habitats in which it is introduced since it drastically changes communities. In Portugal, *Acacia* spp. is one of the most prolific plant invaders, specifically the species *Acacia longifolia*, introduced in the country's coastal areas in the late 19th century, early 20th century. Since their introduction, the species has colonized agroflorestal and dune ecosystems, disrupting their functioning, altering the balance in native flora community. One of the key functional traits of invasive potential and colonizing capacity of these alien species lies in their ability to perform biological atmospheric nitrogen fixation. The nitrogen fixation occurs inside of newly formed organs, the nodules, as a result of a symbiosis established between the plant and legume nodulating bacteria, the rhizobia. The process of biological nitrogen fixation and the impact that Acacia spp. have on the habitats it invades, specially concerning the soil and the above and belowground communities is well documented. However, not much is known about how the characteristics of the soil itself, as well as other abiotic factors, might affect plant development and its influence on the process underlying the species invasive ability. This study intends (1) to access how abiotic factors modulate *A. longifolia* development, using seedlings grown in three different types of soil – forest, agricultural and dune, and submitted to different conditions of irrigation and nutrition. We set out to evaluate the effect of these factors on overall plant development, and (2) to correlate also plant development with the plant capacity to nodulate. Furthermore, we intent (3) to better understand the symbiosis between nitrogen-fixing microorganisms, as well as, (4) to find the correlation between SPAD readings and photosynthetic pigments content, through the determination of the calibration equation. Seedlings of *A. longifolia* were germinated in the different types of soil (forest, agricultural and dune) under four treatments – hydric comfort *vs* hydric stress, in combination with presence *vs* absence of nutritive solution. After twenty weeks of growth, performance of the plants was evaluated by measuring (1) shoot and root length; (2) total leaf area; (3) number and fresh weight of nodules, phyllode and roots and (4) relative levels of chlorophyll through SPAD. Later, absolute photosynthetic pigments content was determined, while the nitrogen fixation process was evaluated based on the isotopic composition of carbon and nitrogen, both in nodules and phyllodes. The results demonstrate that specimens germinated in forest and agricultural soil grew more, but the ones in the latter had fewer nodules. In turn, *A. longifolia* seedlings developed in dune soil had a lower growth and a greater development of root nodules. Furthermore, a higher nutrient availability led to a greater plant development, accompanied by less nodulation and nitrogen fixation. Though, with the reduction of nutrient reinforcement, there was a greater fixation of atmospheric nitrogen, since phyllode $\delta^{15}N$ values were closer to zero in the absence of nutrient solution. This trend, transversal to soil type, was particularly evident in the dune soil. Thus, our results indicate not only the preponderance of abiotic factors such as soil and nutrition in the promotion and hinder of growth and nodulation of *Acacia longifolia*, but also that this conditioning is a result of their interaction. Regarding the calibration between the SPAD readings and the absolute levels of photosynthetic pigments, despite the low adjustment coefficients, the calibration equations obtained had predictive capacity. However it's necessary to analyse more samples to validate the model. Rhizobia analysis indicated not only, the varied and heterogeneous bacterial community associated with *A. longifolia*, but also the species status as a generalist. However, it was not possible to establish a correlation between the number and diversity of isolates and the treatments applied, since strains from the same soil and treatment combinations grouped into different clusters. Additional studies are necessary in order to identify the isolated bacteria and better understand the rhizobial community associated with *A. longifolia* and its influence on plant fitness.In conclusion, *Acacia longifolia* is an extremely aggressive invasive species that disturbs and modifies habitats and communities both in Portugal and in other regions of the world, rendering it essential the study of the factors that influence the development of the species.

Key words: *Acacia longifolia*, abiotic factors, nodules, bacteria, nitrogen fixation

Table of Content

List of Figures

List of Tables

1.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 can additionally have human and economic impacts, since the biological invader might also affect agriculture and forestry (e.g. Brockwell *et al*. 2005; Richardson *et al*. 2011).

1.1 *Acacia longifolia* – functional traits

The genus Acacia is amongst the most aggressive invaders worldwide (Brockwell *et al*. 2005). This group of leguminous woody plants causes severe problems in habitats in which it is introduced since it drastically changes communities, both above and belowground, causing changes in soil, notably in the carbon and nitrogen reservoirs, as well as in the water cycle (Brokwell *et al*. 2005; Marchante *et al*. 2015; Duarte *et al*. 2016). An important competitive advantage of most acacias in relation to native plants results from the possibility of establishing mutualism with nitrogen fixing bacteria present in the soil that promote their nutrition and growth (Brokwell *et al*. 2005). Therefore, the introduction of the genus Acacia diminishes biological diversity and alters the functioning of ecosystems, decreasing their value and causing severe damage to their functionality (Ulm *et al*. 2017; Marchante *et al*. 2015; Rascher *et al*. 2011b; Hellman *et al*. 2011).

In Portugal, *Acacia* spp. is one of the most prolific plant invaders, specifically the species *Acacia longifolia* (Duarte *et al*. 2016). *A. longifolia* (Andrews) Wild., is a shrub (or small tree) belonging to the subfamily Mimosoideae, included in the family Fabaceae (Leguminosae). This species is original from southeast Australia and it was introduced in Portugal, in coastal areas, in the late 19th century, early 20th century by the forestry services with the aim of stabilizing the dunes and controlling its erosion (Marchante *et al*. 2008). Later, it was introduced in Vila Nova de Milfontes in the late 60's, early 70's (Vicente, 2016) and currently it is present in most of the Portuguese coast (Fig. 1.1).

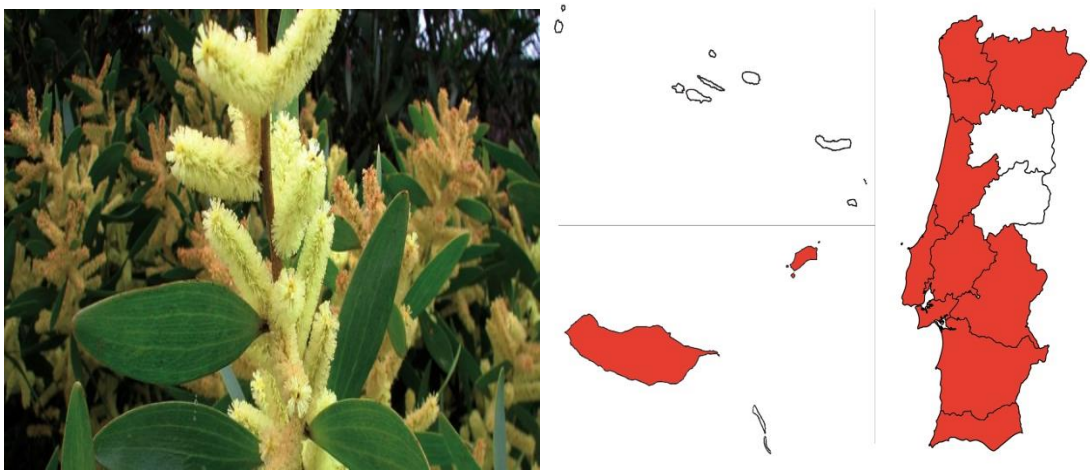

Figure 1.1: Left- Close up of *A. longifolia* phyllodes and inflorescences; Right- *A. longifolia* distribution in Portugal. Retrieved from: http://invasoras.pt/en/gallery/acacia-longifolia-en/ (22/03/2019).

This species can be considered as an ecosystem engineer (Richardson *et al*. 2000; Marchante *et al*. 2011). Following introduction, *A. longifolia* alters nutrient cycles, water availability, community composition and might even disrupt fire regimes (Marchante *et al*. 2008; Racher *et al*. 2009; Hellman *et al*. 2011; Le Maitre *et al*. 2011). Its high growth rate and the presence of evergreen phyllodes, lead to the formation of dense canopies (Le Maitre *et al*. 2011), which limits the availability of light to understory plants (Rascher *et al*. 2011b; Souza-Alonso *et al*. 2017). This decrease in illumination will affect under canopy species and thus reduce species diversity. In addition, this invasive species also has shape plasticity, adapting to available height class niches, adopting shrub or small tree forms (Rascher *et al*. 2011a, b). The species invasive success is also due to the strategies adopted by the plant during 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 use of alternative water sources and root systems to the native flora (Antunes *et al*. 2018).

Acacia longifolia is also characterized by producing a large number of seeds that can accumulate under the canopy, to be dispersed by other living beings, thus having the growth and reproduction facilitated in comparison with the native species of the habitat in which it is introduced (Marchante *et al*. 2015; Rascher *et al*. 2011b; Marchante *et al*. 2008).The seeds of *A. longifolia* have characteristics that favour their invasive capacity, namely they have a hard integument, persist in the soil, suffer less pre-dispersion predation, are of a large size and are heat tolerant (Correia *et al*. 2015, 2016). Its germination is even stimulated by heat and / or smoke (Marchante *et al*. 2008). Therefore, the fires, which have a high occurrence in Portuguese soils, are an important factor for the dispersal and growth of *A. longifolia*.

Additionally, we frequently observe an accumulation of litter, which contributes to the alteration of soil characteristics and nutrient cycles. Biomass accumulation increases organic matter content, as well as nitrogen and carbon pools, which at first could seem to benefit native species. However, local flora cannot compete with *A. longifolia* growth rates and furthermore the species phosphorus requirements disrupt N / P balance (Hellman *et al*. 2011; Souza-Alonso *et al*. 2017; Ulm *et al*. 2017).

One of the key functional traits that ensure *Acacia* spp. invasive success is their ability to perform biological atmospheric nitrogen fixation, which consists in the conversion of gaseous atmospheric nitrogen into ammonia by the action of an enzyme (nitrogenase) (Dupont *et al*. 2012). The nitrogen fixation occurs inside of newly formed organs, the nodules, primarily in the roots of the plant, again, as a result of a symbiosis established between the plant and legume nodulating bacteria, collectively called rhizobia.

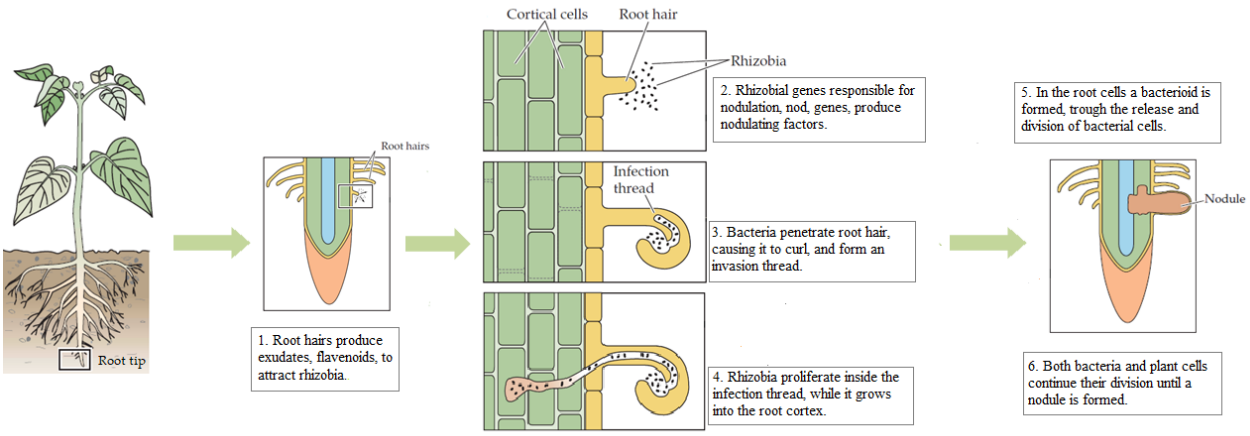

Figure 1.2: Stages of root nodule formation in a leguminous plant, adapted from Sadava *et al*. 2014.

This is a mutual beneficial relation since the bacteria receive from the plant dicarboxylic acids as a source of carbon and in return the plant receives ammonium form the rhizobia (Brockwell *et al*. 2005). The nodulating bacteria might belong to at least eight different genera *Rhizobium, Bradyrhizobium,* particularly *Bradyrhizobium japonicum, Sinorhizobium, Mesorhizobium, Azorhizobium* and *Allorhizobium*, as well as *Ochrobactrum and Ensifer melitoti* (Brockwell *et al*. 2005; Rodríguez-Echeverría *et al*. 2011; Souza-Alonso *et al*. 2017) and the same species and eventually the same tree are able to form nodules with bacteria of several taxonomic groups (Brockwell *et al*. 2005). The origin of these symbionts is still debated. Some studies indicate that *Acacia* spp. establish symbiotic relationships with co- introduced microbes, while others suggest that new mutualisms are established in the invaded locations (Rodríguez-Echeverría *et al*. 2011; Souza-Alonso *et al*. 2017).

Moreover, there are indications, still little explored, that new symbiotic relations might occur due to the horizontal transfer of symbiotic genes (responsible for nodulation and nitrogen fixation), since they are present in plasmids and other transposable elements (De Meyer *et al*. 2015), to bacteria present in the soil (Rodríguez-Echeverría *et al*. 2003, 2010; Ndlovu *et al*. 2013; Rout *et al*. 2012). Also, there are indications, that not all bacteria present in nodules effectively perform nitrogen fixation, non-symbiotic endophytic bacteria, which function is still uncertain, seem to promote growth (Fteich *at al*. 2012; Ndlouv *et al*. 2013; De Meyer *et al*. 2015). 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).

1.2 Stable isotopes

Isotopic analysis is a powerful tool in understanding the original source of nitrogen in the plant, and to what extent fixation is an important contribution to the nitrogen pool in plant cells. Stable isotopes are tracers of ecological processes, resulting from the interaction between plants and environment. It's an empirical tool to better comprehend how plants are conditioned, and how several processes are mediated by biotic and abiotic factors. For instance, determination of N_2 source and contribution of nitrogen fixation in several plant tissues can be achieved through $\delta^{15}N$ index analysis. Atmospheric nitrogen $\delta^{15}N$ is considered the standard in terms of mass spectrometry, with a value of 0‰. Since soil ^{15}N content is higher than atmospheric content, when comparing plant tissue samples with atmospheric N_2, lower, near zero or negative, values are indicative of nitrogen fixation (Rodríguez-Echeverría *et al*. 2007; Dawson *et al*. 2002).

Moreover, ^{13}C signature is another useful tracer of ecological processes. The analysis of $\delta^{13}C$ is a valuable indicative of parameters such as water use efficiency, and also of carbon distribution to different plant organs, being also instrumental in its quantification (Dawson *et al*. 2002).

1.3 SPAD

Another indicator of plant health and physiological status are photosynthetic pigments content, which are affected by both biotic and abiotic factors, commonly used in plant ecological studies (Udling *et al*. 2007; Ling *et al*. 2011). However, determination of photosynthetic pigments content is a time consuming and laborious process, involving the destruction of plant material. An alternative

method is the use of SPAD (soil plant analysis development) meter. This portable reader determines relative leaf pigment concentrations through measurements performed using two wavelengths, 650 nm and 940 nm, respectively in the red and infrared regions of the electromagnetic spectrum. However, these measurements are species specific and so to convert relative readings to absolute pigment concentrations, there is the need to establish a calibration curve, significantly representing this correlation (Ling *et al*. 2011). This method has been successfully established for other species, such as *Arabidopsis thaliana* (Ling *et al*. 2011). A calibration curve was also determined for birch, wheat and potato (Uddling *et al*. 2007).

The process of biological nitrogen fixation and the impact that Acacia spp. have on the habitats it invades, specially concerning the soil and the above and belowground communities is well documented (Souza-Alonzo *et al*. 2014; Marchante *et al*. 2008; Hellman *et al*. 2011). *A. longifolia* alters soil properties, namely pH and nitrogen and carbon pools. Furthermore, this species progressively disrupts nutrient dynamics, water cycle and soil microbial communities (idem).

However, not much is known about how the characteristics of the soil itself, as well as other abiotic factors, might affect plant development and its influence on the process underlying the species invasive ability.

1.4 Aims

Considering this, our study intended (1) to access how abiotic factors modulate *A. longifolia* development, using seedlings grown in three different types of soil – forest, agricultural and dune, and submitted to different conditions of irrigation and nutrition. We set out to evaluate the effect of these factors on overall plant development, and (2) to correlate also plant development with the plant capacity to nodulate. Furthermore, we propose (3) to better understand the symbiosis between nitrogen-fixing microorganisms, such as *Rhizobium* and others, and *A. longifolia*, not only in terms of bacterial diversity, but also how the factors previously mentioned might affect the establishment and growth of nodules. In this study, we also intent (4) to find the correlation between SPAD readings and photosynthetic pigments content, through the determination of the calibration equation.

2. Materials and Methods

2.1 Seed collection and germination

Matured pods of *Acacia longifolia* were collected in Vila Nova de Milfontes (Odemira, Portugal), from trees growing in agricultural, forest and dune soil, in July 2017.

After collection, the seeds were manually removed from the pods and stored at room temperature until germination. The seeds were surface sterilized with pure commercial bleach (containing sodium hypochlorite) for 5 min, rinsed with distilled water and subjected to a water bath, at 100ºC, for 1 min. Seeds were then placed in Petri dishes and incubated for 48h in darkness followed by a 16h light photoperiod for two weeks, before being transferred to the greenhouse.

2.2 Soil collection and analysis

Soil samples from agricultural, forest and dune soils were collected in the vicinity of the acacias, in September of 2017. The samples were sieved with a 40mm sieve, manually homogenized and sent for analysis for texture, pH, organic matter, nitrate-N, ammonium-N, potassium, sodium, calcium, magnesium and phosphorus at Laboratório de Análises de Solos e Plantas, UTAD – Universidade de Trás-os-Montes e Alto Douro. Analyses were performed in triplicate. The obtained results are summarized in the table 3.1.

2.3 Experimental set-up

After germination, the growing seedlings were individually transplanted to plastic dark pots containing one of the three soils (forest, agricultural and dune) and transferred to the greenhouse, where they were maintained under natural photoperiod. Temperature was not controlled, but the ventilation system assured it was never above 25°C.

Following a two weeks acclimation, where all plants in the 3 types of soil were maintained at optimal hydric conditions, the length of the plants was measured and the experimental period was initiated. For each soil type (forest, agricultural and dune), four treatments were applied – hydric comfort *vs* hydric stress, in combination with presence *vs* absence of nutritive solution (Fig. 2.1). In the experiment, each treatment had 10 replicates, in a total of 40 plants in each type of soil, in a randomized design.

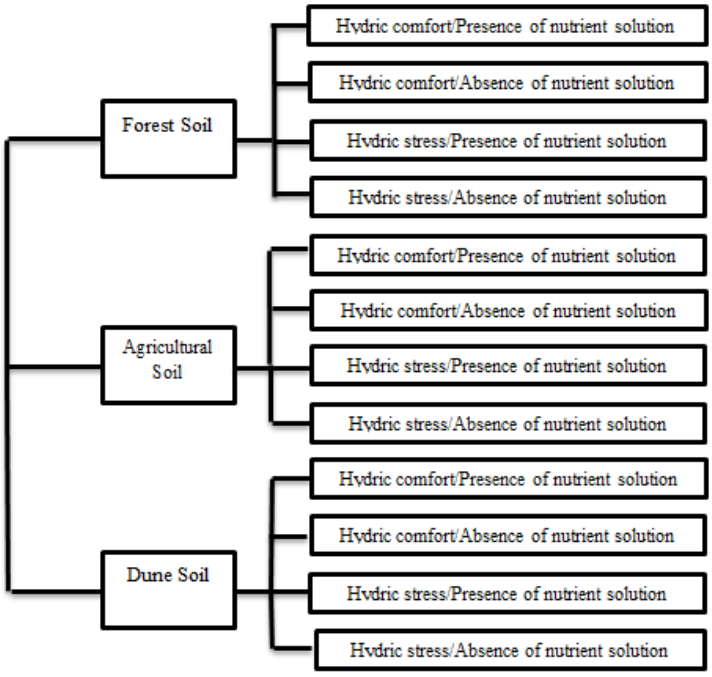

Figure 2.1: Experimental Design. All treatments were applied to the three types of soil, each with ten replicates.

The hydric comfort is related to the amount of water added, which was determined according to field capacity. The plants in a water comfort regime were maintained at 70% field capacity, corresponding to 35 mL of tap water (or nutrient solution, depending on the treatment applied). Hydric stress was applied by reducing the water to 30% of the field capacity, corresponding to 15 mL. Regardless of the treatment all plants were watered twice a week. Nutrient solution composition when added was based on the Hoagland Solution (in Appendix I, Table 6.1).

For easier reading and understanding, the treatments will be referred as described in Table 2.1.

Table 2.1: Treatment description and abbreviations applied in all three soils. W+ represents hydric comfort; W-, hydric stress; N+, presence of nutrient solution and N-, absence of nutrient solution.

Treatment Abbreviation	Treatment	Composition
W+N+	Water at 70% field capacity/Presence of nutrient solution	35 mL of nutrient solution
W+N-	Water at 70% field capacity /Absence of nutrient solution	35 mL of water
W-N+	Water at 30% field capacity /Presence of nutrient solution	15 mL of nutrient solution
W-N-	Water at 30% field capacity /Absence of nutrient solution	15 mL of water

The plants were grown in the greenhouse following this experimental set-up for 20 weeks, from November 2017 to April 2018. After this period, the trial was evaluated.

2.4 Data Collection

2.4.1 Physiological performance evaluation

Following the 20-weeks growth period, eight out of the ten replicate plants, of each treatment, were removed from the pots.

In each individual plant a set of parameters was measured as depicted in Fig. 2.2 and Fig. 2.5: (1) shoot and root length; (2) number and fresh weigh of root nodules, phyllodes and roots; (3) total leaf area and (4) relative chlorophyll levels, for two leaf regions, I and II, in one phyllode per plant, as depicted in Fig.2.2.

For (3), a LI-3100C Area Meter was used, while (4) used a hand-held chlorophyll meter SPAD-502. Phyllodes, roots and nodule samples were collected, and oven dried at 120ºC for at least 48h for dry weight measurement.

Based on these measurements, plant growth; root/shoot ratio and water content of roots and phyllodes was calculated with the following equations, respectively; (1) Shoot Increment = Final Length – Initial Length; (2) Root/Shoot Length Ratio = Root Length/Final Shoot Length; (3) Root/Shoot Fresh Weight Ratio = Root Fresh Weight/Shoot Fresh Weight; (4) Phyllode Hydric Content = Phyllode Fresh Weight – Phyllode Dry Weight and (5) Root Hydric Content = Root Fresh Weight – Root Dry Weight.

Figure 2.2: *Acacia longifolia* pyllode regions (I- Phyllode base; II- Phyllode tip) in which relative chlorophyll was measured with SPAD-502 and sections were removed determination of photosynthetic pigments content.

2.4.2 Photosynthetic pigment determination

Additionally, photosynthetic pigments content was evaluated. Three 0.5cm diameter discs were removed from the same two regions (I and II) from a phyllode in five of the eight replicates, with the aid of a manual punch. The discs were then suspended in 2mL of methanol and stored at 4°C in the dark, for 48h. After this, absorbances were measured at 470nm, 652.4nm and 665.2nm with Thermo Heλios β spectrophotometer (Thermo Electron Coorporation). Chlorophyll a, b, total of chlorophylls and total of carotenoids concentrations (in μg/mL) were calculated according to the equations below (Fig. 2.3).

$$C_a = 16.672A_{665.2} - 9.16A_{652.4}$$

$$C_b = 34.09A_{652.4} - 15.28A_{665.2}$$

$$C_{a+b} = 1.44A665.2 + 24.93A_{652.4}$$

$$C_{\chi+c} = \frac{1000A_{470} - 1.63C_a - 104.96C_b}{221}$$

Figure 2.3: Equations for the determinations of the concentrations of chlorophyll *a* (C_a), chlorophyll *b* (C_b), total chlorophylls (C_{a+b}) and total carotenoids ($C_{\chi+c}$) (Lichtenthater, 1987).

Correlation between values obtained through SPAD and chlorophyll determination was calculated for *A. longifolia* phyllodes.

2.4.3 Carbon and Nitrogen Isotopic Analysis

Isotopic studies were performed, using an average of two phyllodes and five nodules of three plants selected of each soil and treatment. The samples were oven dried at 120ºC for 48h. Following mechanical maceration with a ball mill (Retsch, Haan, Germany), 5mg of phyllodes and 1mg of nodules of the resulting fine powder were weighed and encapsulated into tin capsules. The samples were then sent for analysis for $\delta^{15}N$, $\delta^{13}C$, nitrogen (%N) and carbon (%C) content at LIE-SIIAF (Stable Isotopes and Instrumental Analysis Facility, at Faculdade de Ciências, Universidade de Lisboa).

2.5 Nodules and Bacteria

The nodules were collected from the roots for microbiological diversity analysis. Nodules from the same treatment and soil were grouped and kept in a microtube with distilled water.

The first step of the analysis consisted in the disinfection of the nodules (Fig.2.4) to prevent contamination from soil bacteria, ensuring that the isolated organisms were from the nodule (inside). All the culture media used for bacterial isolation was previously sterilized by autoclaving at 121°C for 20 minutes, before pouring it to sterile Petri dishes.

To confirm that the nodule surface was in fact disinfected, prints of the nodules were made in fresh plates of Yeast Mannitol medium (in Appendix I, Table 6.2), followed by incubation at 28 °C for 72 h. If no growth was observed, the nodules were macerated using a sterile mortar and pestle and resuspended in 300µL NaCl 0.85%.

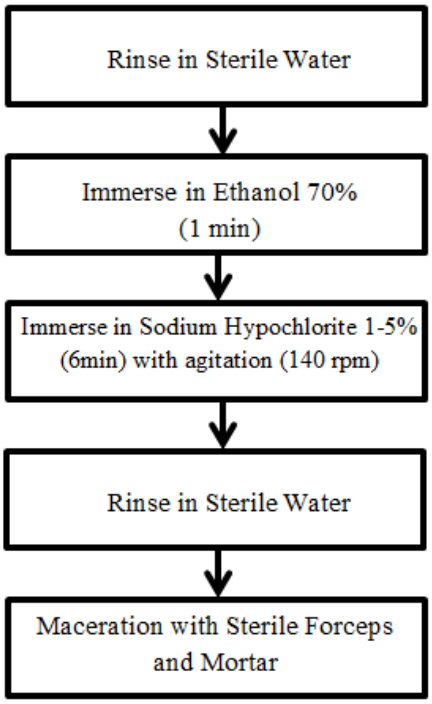

Figure 2.4: Nodule sterilization protocol, adapted from Hungria *et al*. (2016).

Of those, 250 µL were inoculated in YMA (Yeast Mannitol Agar) medium (in Appendix I, Table 6.2) with 0.02 gL^{-1} of cycloheximide to avoid fungal contaminations. The Petri dishes were incubated at 28 ° C, and growth was observed after 48 to 72 hours.

The resulting single colonies were restreaked three times to achieve purification. The colonies were then observed macroscopically and with a magnifier to discern shape and overall appearance. Microscopic observation, followed by Gram staining, allowed the characterization and differentiation of the isolates according to type of bacteria and Gram staining, as well as the verification of colony purity.

The Gram staining procedure followed consisted in (1) the smear of a culture scoop in a water drop over a slide; (2) heat fixing; (3) addition of crystal violet for 1 min; (4) rinse with tap water; (5) addition of iodine solution for 1 min; (6) rinse with tap water; (7) addition of alcohol for 20 sec; (8) addition of safranin for 1 min; (9) rinse with tap water (Bartholomew *et al*. 1952).

The non-pure colonies were suspended in 100 ml of distilled water, pasteurized at 80ºC for 15 minutes and inoculated again, according to the previously stated method.

Lastly, biochemical analyses were performed for oxidase, KOH and catalase reactions, to further characterize the isolates. For oxidase, an isolated colony was removed and smeared over filtered paper soaked in 1% tetramethyl-p-phenylenediamine, over a slide, while for KOH test a colony was removed with a loop and mixed with a drop of 3% KOH, over a slide. As for catalase test, a loop of isolated colony was mixed with a drop of commercial hydrogen peroxide over a slide (Madigan *et al*. 2017).

2.6 DNA Extraction and Fingerprinting

DNA extraction was carried out by the boiling method, following a protocol provided by BioISI, as follows: one to three colonies were removed with a loop, 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, to discriminate between different species of microorganisms. Three different primers were tested - M13, PH and GTG5, in all samples obtained. All amplification reactions were performed in a total volume of 25 µL - 18.3 µL of water, 2.5 µL of 10xInvitrogen buffer; 1.5 µL of 50mM $MgCl_2$; 0.5 µL dNTP mix; 0.3 µL of primer; 0.2 µL of 5U/µL Invitrogen Taq DNA polymerase 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 5min at 95ºC, followed by 40 cycles of 1min at 95ºC, 2min at 50ºC, 2min at 72ºC and a final extension of 5min at 72ºC.

The products of amplification were subsequently analysed using 1.2% agarose gel electrophoresis at 90V for 3 hours, 0.5xTBE being used as the gel and running buffer. The gels were then stained with ethidium bromide solution for 15 minutes and visualized through UV transilluminator Alliance 4.7 (Uvitec, Cambridge).

2.7 Statistical Analysis

The data was analysed using IBM SPSS Statistics 25.0 (SPSS, Inc., Chicago, IL, United States). Data normality and the homogeneity of variances were accessed. When the variables didn't fulfil the assumptions, the data set was considered to have a non-normal distribution. The relationship between the response variables was accessed through Spearman correlation. For simplification, in situations where variables were found with a correlation index of 0.7 or higher, one of the variables was represented by the other and it was excluded from the analysis.

Lastly, the data were subjected to an analysis of variances. Kruskall-Wallis test and post-hoc comparisons, with treatment combinations as factors, were conducted to determine their effect on the response variables.

For the bacterial DNA fingerprinting, the images resulting from gel observation were analysed with Bionumerics (Applied Maths NV) software to obtain the dendrograms with Pearsons' coefficient.

Figure 2.5: Data collection. (a) Experimental set-up at the end of the 20-week experimental period; (b, c) Removal of plants from the pots; (d) Measurement of chlorophyll relative content with SPAD-502; (e) Leaf Area Meter; (f) Removal of phyllode discs for photosynthetic pigments content determination; (g) Measurement of plant components; (h) Removal of root nodules; (i) Examples of root nodules.

3. Results

3.1 Soil Analysis

The soils were characterized by texture, pH, organic matter percentage, electric conductivity and nutrient availability (Table 3.1). While all three soils had similar coarse texture, forest soil was more acidic, with a pH=4.8, whereas agricultural and dune soils were more moderately acidic, with pH values around 6. The highest organic matter content (2.16%) was obtained in forest soil, while agricultural and dune soils showed a percentage of approximately 0.4 and 1 percent, respectively. In contrast, forest soil presented the lowest electric conductivity, around 0,05dS m^{-1}, compared with dune and agricultural soils, with values of 0.26 and 0.12 dS m^{-1}, respectively.

A pattern could be discerned among the soils regarding nitrate-N (64.27 mg element kg^{-1}), potassium (141.31 mg element kg^{-1}), sodium (58.97 mg element kg^{-1}), calcium (782.95 mg element kg^{-1}) and magnesium (385.7 mg element kg^{-1}) availability, where the highest values were found in agricultural soil and decreased in dune and forest soil. However, ammonium-N and phosphorus were exceptions to this pattern. The availability of N-NO4 was the lowest in agricultural soil (0.33 mg element kg^{-1}), followed by forest and dune soils with similar nutrient content, respectively, 1.57 and 1.27 mg element kg^{-1}, while the highest values of available phosphorus were measured in agricultural soil (12.5 mg element kg^{-1}), while forest and dune soils had lower values, 0.72 and 0.22 mg element kg^{-1}, respectively.

Table 3.1: Soil characterization according to texture, pH in water, organic matter content, electric conductivity and nutrient availability.

Type of Soil	Texture	pH water	Organic matter (%)	Electric conductivity (1:5) dS m^{-1}	Extraction in water (1:5) - mg element kg^{-1}						
					N-NO3	N-NO4	P	K	Na	Ca	Mg
Forest	coarse	4,750	2,160	0,046	1,250	1,568	0,723	23,205	9,386	91,215	64,698
Agricultural	coarse	6,045	0,395	0,257	64,268	0,328	12,499	141,306	58,971	782,945	385,698
Dune	coarse	6,055	1,039	0,122	26,685	1,268	0,218	41,903	49,746	319,330	176,168

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3.2 Overall assessment of treatment combinations on plant development

In what concerns data analysis, the conducted exploratory analysis of normality conducted, considering as factors the treatments and as dependent variables the parameters measured following the experimental period, concluded that the data did not have a normal distribution. Additionally, Spearman's correlation found significance between number of phyllodes and leaf area; phyllode fresh and dry weight; C_{a+b} content and C_a and C_b content both from the base and from the tip of the phyllode. Therefore the parameters number of phyllodes, phyllode fresh weight, C_{a+b} content from the base and tip of the phyllodes became representatives of the variables with which they were correlated, when statistical analysis was concerned.

3.2.1 Increment, roots and root/shoot ratio

When considering all the treatments (Fig. 3.1), analysis showed that plants grown in the combination forest soil (F), water at 70% field capacity (W+) and nutrient solution (N+), (FW+N+) had on average a higher increment in shoot size, of 57 cm after 20 weeks, although, similar growth values were registered in treatments in conjunction with agricultural soil. Statistically significant differences were found between FW+N+ and the treatment combinations dune soil (D), water at 70% field capacity (W+) and absence of nutrient solution (N-) (DW+N-), in which the lowest mean increment was registered, with 32.34 cm. (Fig. 3.2 and Appendix II).

Figure 3.1: *Acacia longifolia* growth at the end of the experimental period of 20 weeks, before removal from pot.

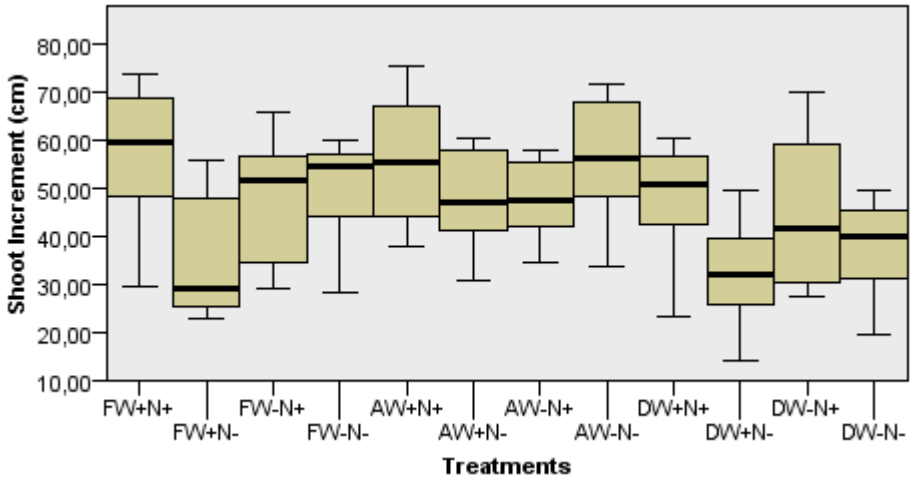


Figure 3.2: Variance analysis box-plot of shoot increment (cm) in plants of *A. longifolia*, measured after a growth period of 20 weeks with different treatments (n=96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

In what concerns root length (Fig. 3.3), similar results were found. Plants grown in the treatment combination FW+N+ presented longer roots, while lower values were registered in the combination DW+N- (Fig. 3.4). In spite the of the Kruskall-Wallis test finding significance regarding root length, post-hoc comparisons indicated that there were no significant differences between the treatments (in Appendix II).

Figure 3.3: Root length diversity found in *A. longifolia* plants, after a growth period of 20 weeks. Roots from *A. longifolia* grown in agricultural soil at 30% field capacity, without nutrient solution (Left); Roots from *A. longifolia* grown in agricultural soil at 70% field capacity, without nutrient solution (Right).

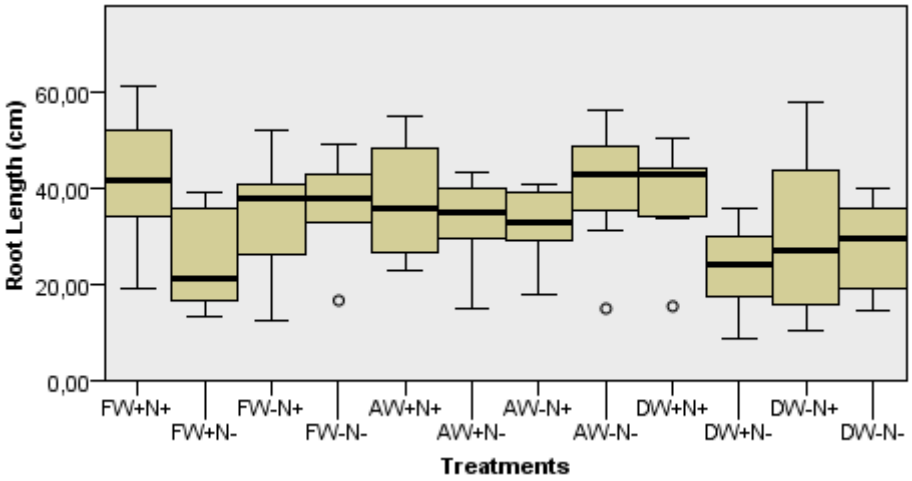


Figure 3.4: Variance analysis box-plot of root length (cm) in plants of *A. longifolia*, measured after a growth period of 20 weeks in plants of *A. longifolia*, measured after a growth period of 20 weeks with different treatments (n=96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

Furthermore, the root/shoot length ratio had similar values with little variance and no statistical significance (Fig. 3.5 and Appendix II), while root/shoot fresh weight ratio registered highest values in DW+N-, with an average of 2.09, with statistically significant differences being found between DW+N- and DW-N+ and FW+N+ (Fig. 3.6 and Appendix II). The roots hydric content, had lower values associated with dune soil, particularly the treatment combination DW+N- , with mean values 0.34. However statistical significance was only found for hydric content, with differences being found between the treatment combinations DW+N- and AW-N-, AW-N+ and AW+N+ (Fig 3.7 and Appendix II).

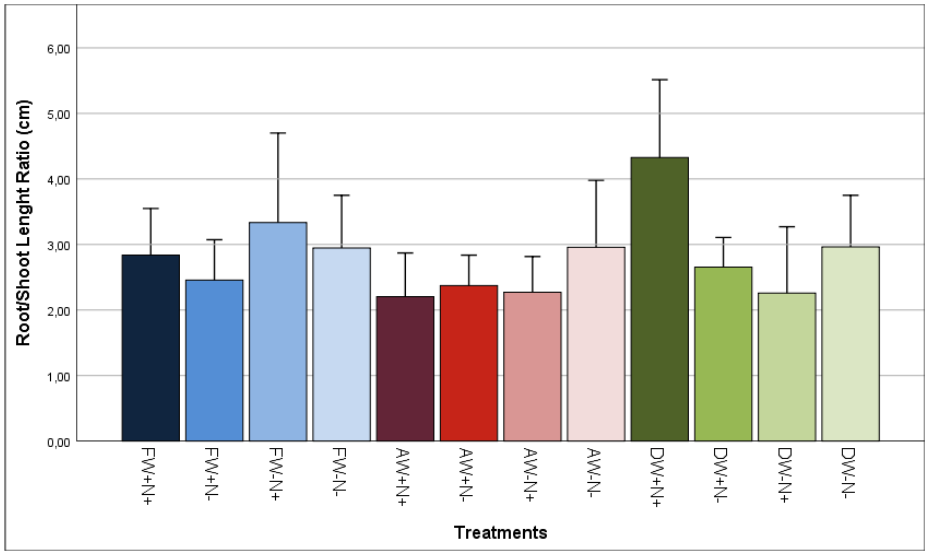


Figure 3.5: Average distribution of root/shoot length ratio (cm) (mean ± 2SE) in plants of *A. longifolia*, measured after a growth period of 20 weeks, according to treatment combination (n= 96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

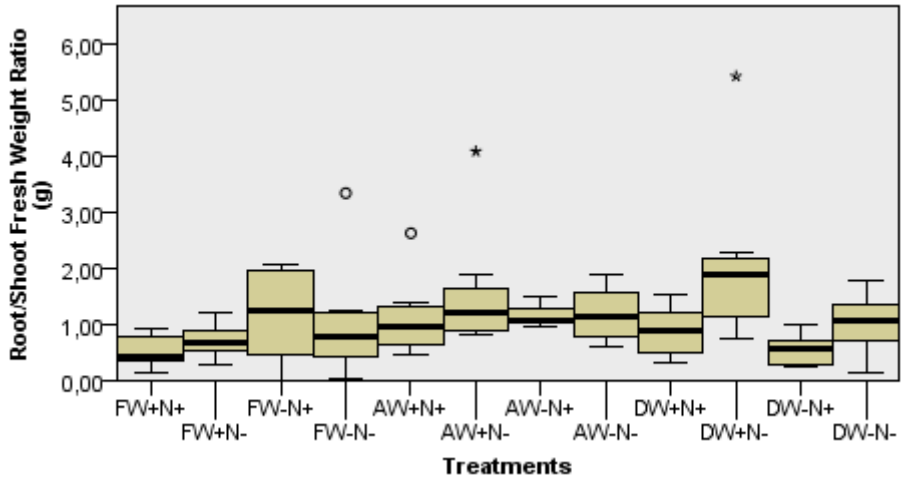


Figure 3.6: Variance analysis box -plot of root/shoot fresh weight ratio in plants of *A. longifolia*, measured after a growth period of 20 weeks (n= 96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution. The symbols represent outliers.

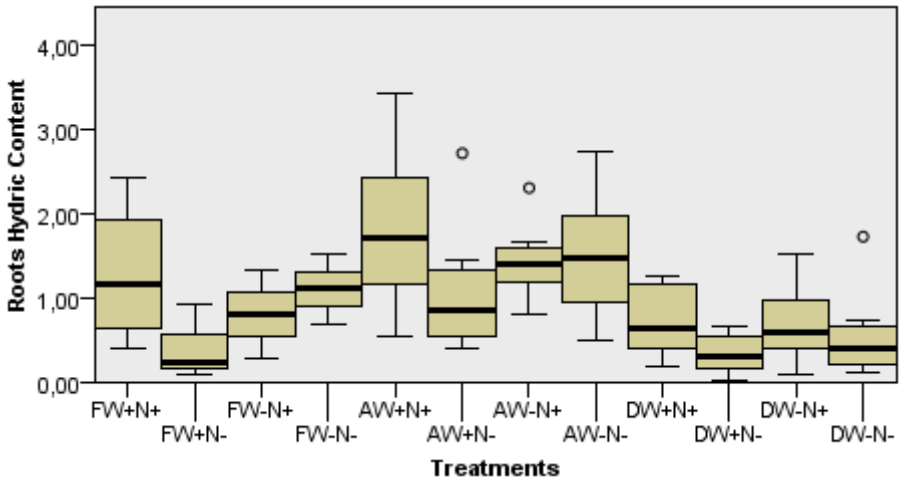


Figure 3.7: Variance analysis box-plot of root hydric content in plants of *A. longifolia*, measured after a growth period of 20 weeks (n= 77). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution. The symbols represent outliers.

3.2.2 Phyllodes

Plants grown in forest soil in conjugation with water at 70% field capacity and nutrient solution (FW+N+) had a higher number of phyllodes (Fig. 3.8), approximately 6 per plant, contrasting with the treatment combination DW+N- which had fewer phyllodes, approximately 1 per plant. Statistically significant differences were found between the combination dune soil (D), water at 70% field capacity (W+) and absence of nutrient solution (N-) (DW+N-) and the treatments AW+N+, FW+N+ and DW-N+ ; and the combination forest soil (F), water at 70% field capacity (W+) and presence of nutrient solution (N+) (FW+N+) and the treatments DW-N- and FW+N- (Fig. 3.9 and Appendix II).

Figure 3. 8: Examples of phyllode diversity (a,b,c,d) found in plants. (a) *A. longifolia* shoot with leaves (small arrow) and phyllodes (large arrow).

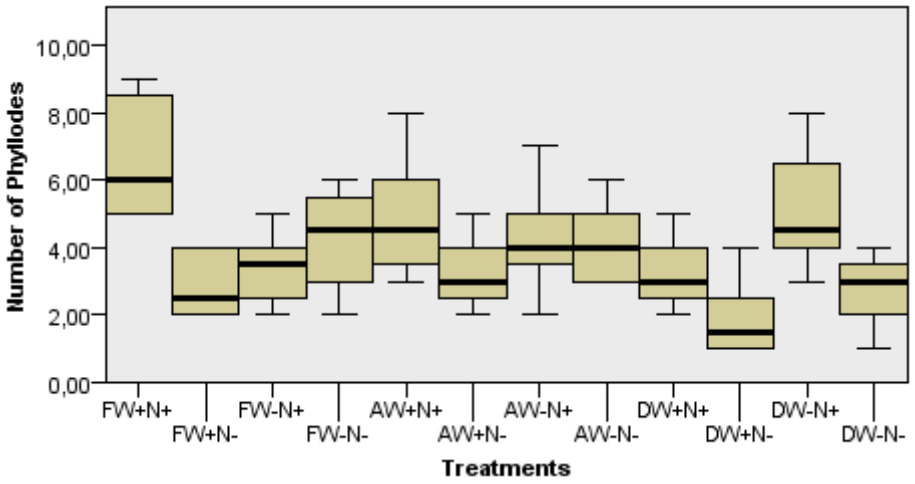


Figure 3.9: Variance analysis box-plot of number of phyllodes in plants of *A. longifolia*, measured after a growth period of 20 weeks (n= 96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

Higher phyllode hydric content was associated with acacias grown with the combination FW-N-, on average 2.8. Statistical analysis revealed significant differences between the combination DW+N- , in which the lowest hydric content was registered, on average 0.20, and the treatments AW-N+, AW-N-, AW+N+, DW-N+ and FW+N+; as well as between the combinations DW-N- and FW+N+ and AW+N+ (Fig. 3.10 and Appendix II).

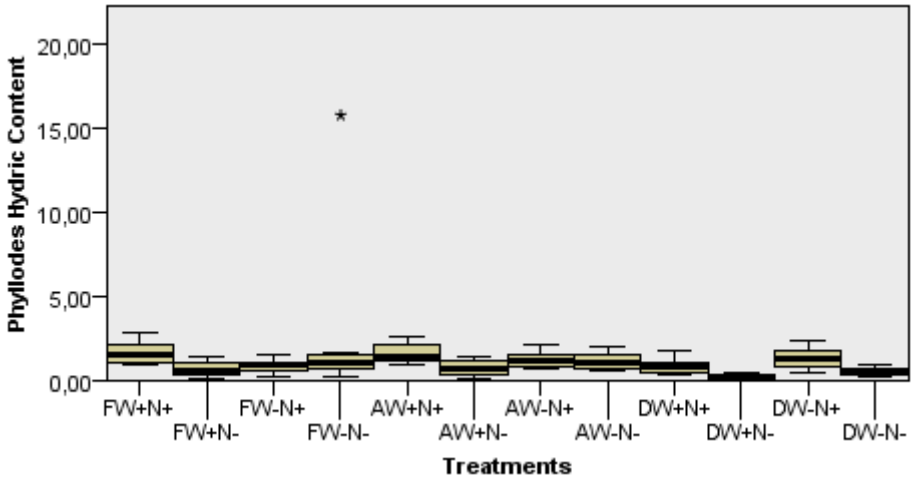


Figure 3.10: Variance analysis box-plot of phyllodes hydric content *A. longifolia*, measured after a growth period of 20 weeks (n= 96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution. Symbols represent outliers.

3.2.3 Nodules

In what regards nodules (Fig. 3.11), a higher number of nodules was registered for the combination dune soil (D), water at 30% field capacity (W-) and presence of nutrient solution (N+) (DW-N+), on average 61 nodules per plant, while the lowest number was registered in plants grown in agricultural soil in association with the combination W+N+, on average 11 nodules per plant.

Furthermore, the combination agricultural soil (A), water at 70% field capacity (W+) and absence of nutrient solution (N-) (AW+N-) registered a higher nodule weight/number ratio with an average of 0.028 (Fig. 3.12). In spite of the Kruskall-Wallis test finding significance in the variables regarding nodule weight/number ratio, post-hoc comparisons indicated that there were no significant differences between the treatments (in Appendix II).

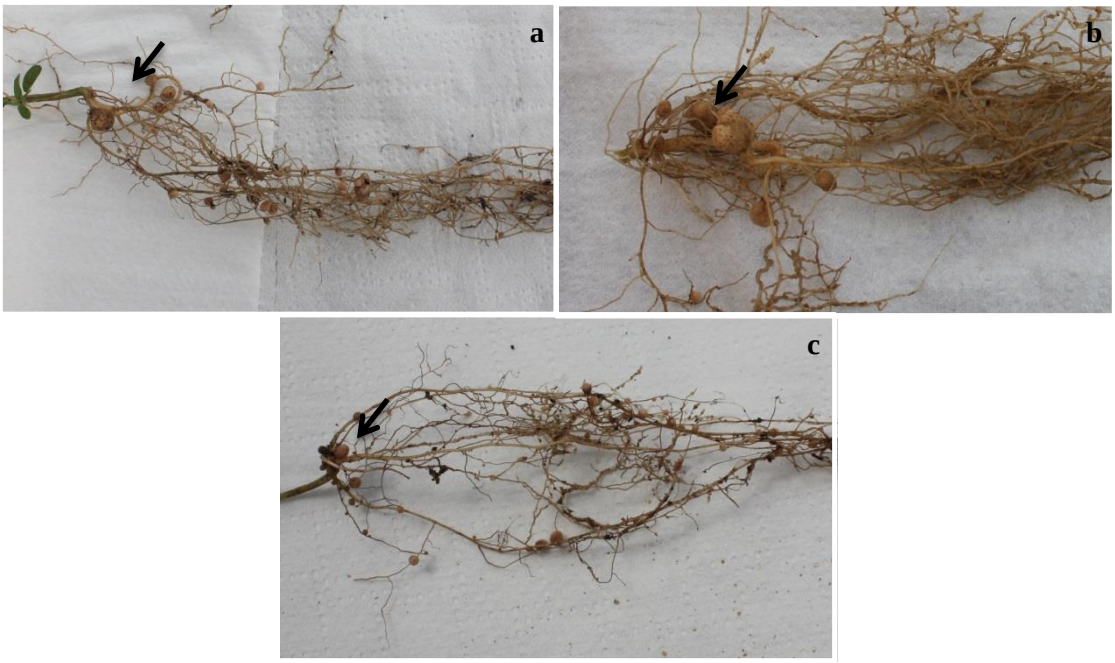


Figure 3.11: Nodules (arrows) found in *A. longifolia* grown in forest (a), agricultural (b) and dune (c) soils showing the variation in size. It is evident that nodules developed in plants in agricultural soils were larger, while present in lower number.

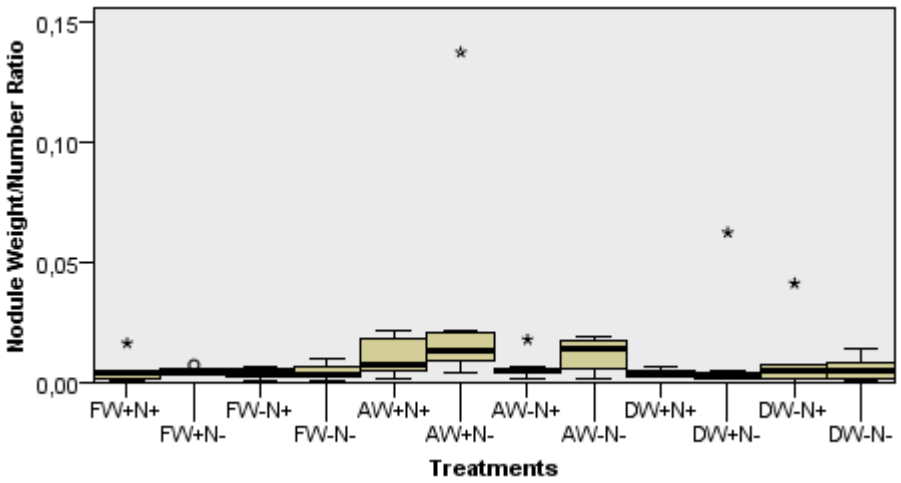


Figure 3.12: Variance analysis box-plot of nodule weight/number ratio in plants *A. longifolia*, measured after a growth period of 20 weeks (n= 96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution. Symbols represent outliers.

Regarding number of nodules, pairwise comparisons showed differences between the treatment combinations FW+N+ and Agricultural soil (A), watering at 70% field capacity, nutrient solution (AW+N+) and agricultural soil, watering at 70% field capacity, absence of nutrients (AW+N-) (Fig. 3.13 and Appendix II).

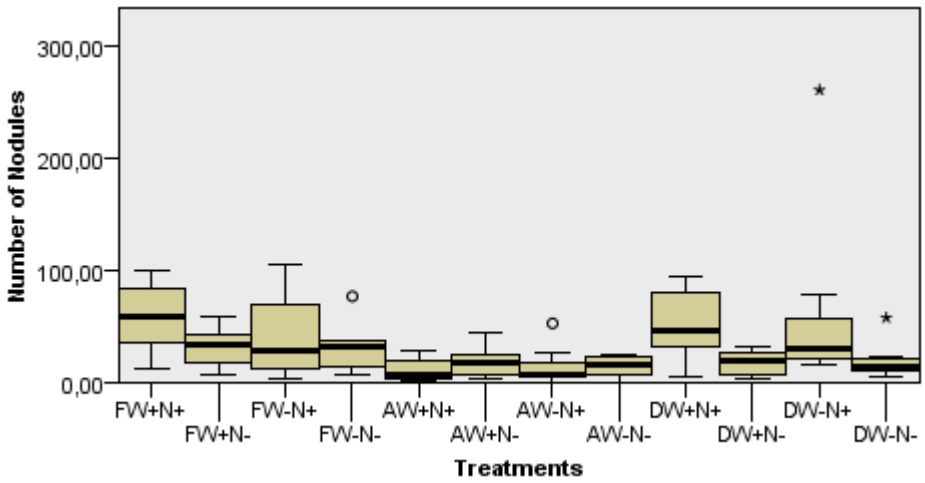


Figure 3.13: Variance analysis box-plot of number of nodules in roots of *A. longifolia*, measured after a growth period of 20 weeks (n= 96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution. Symbols represent outliers.

It was in the agricultural soil, that higher mean nodule weight was registered, in the treatment combination AW+N-, with values of 0.28g. Though, the variable was not statistically significant (Fig. 3.14 and Appendix II).

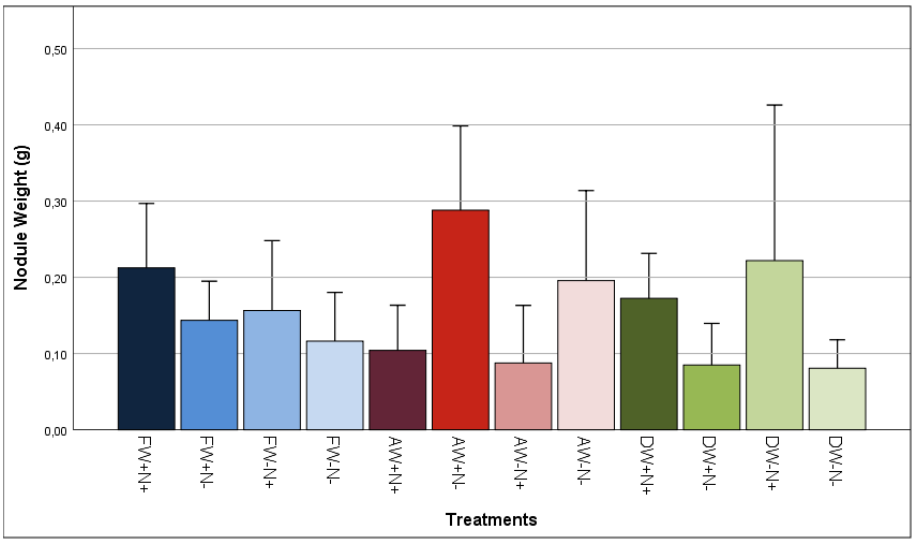


Figure 3.14: Average distribution of nodule weight (mean ± 2SE) of *A. longifolia*, measured after a growth period of 20 weeks, according to treatment combination (n= 96). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

3.2.4 Photosynthetic Pigments Content Analysis

The photosynthetic component was assessed through photosynthetic pigment concentration analysis. The majority of parameters had higher values for DW-N+, namely chlorophyll *a* (C_a) both at phyllode tip and base, chlorophyll *b* (C_b) at phyllode base (Appendix III), total chlorophyll (C_{a+b}) both at phyllode base and tip (Fig 3.15; 3.16) and Carotenoids at phyllode base, with mean values of 32.9, 45.3, 16.6, 43.6, 62.0 and 10.0 mg cm^{-2} respectively.

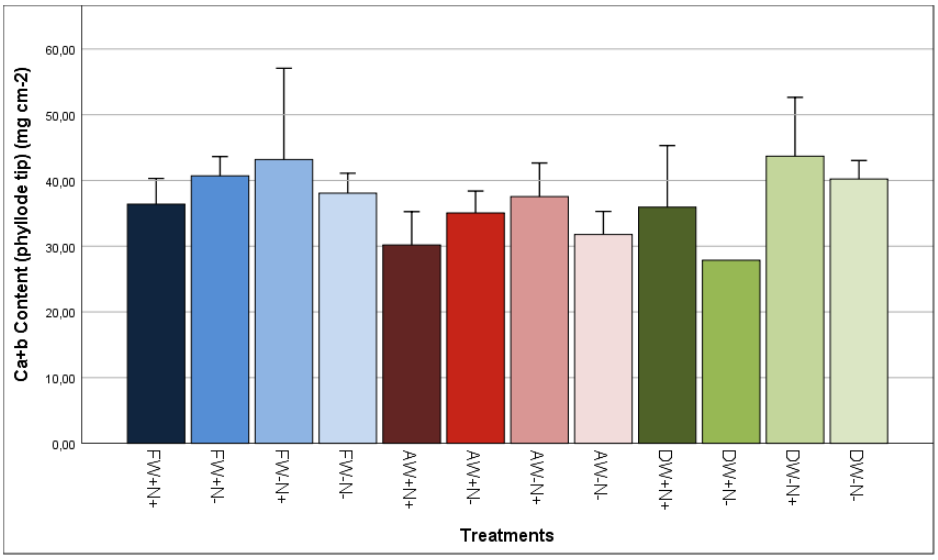


Figure 3.15: Average distribution of C_{a+b} content (phyllode tip) (mean ± 2SE) in plants of *A. longifolia*, measured after a growth period of 20 weeks with different treatments (n=55). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

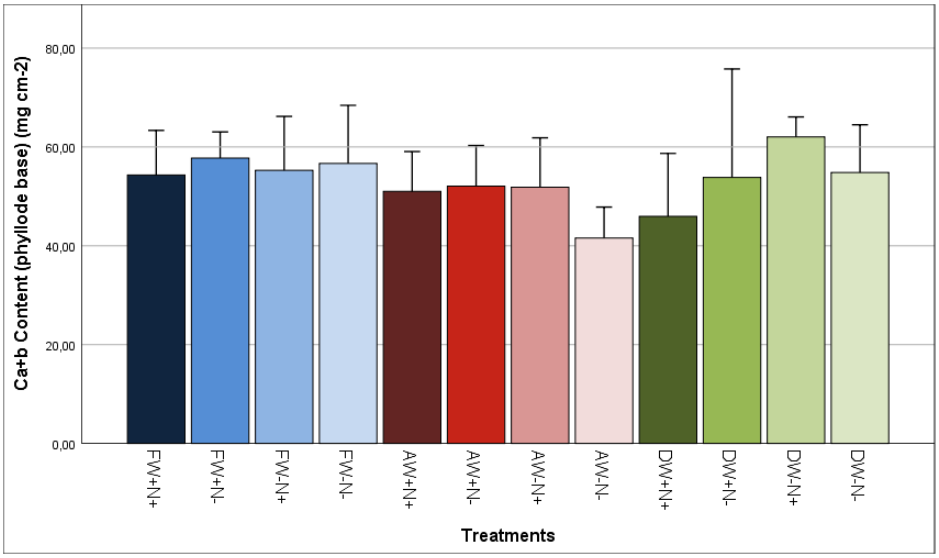


Figure 3.16: Average distribution of C_{a+b} content (phyllode tip) (mean ± 2SE) of *A. longifolia*, according to treatment combination (n=60). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

However, statistically significant differences were only found for carotenoid content, particularly between AW+N+ and FW+N- at phyllode tip (Fig.3.17 and Appendix II) and between AW-N- and DW-N+ and FW+N- at phyllode base (Fig. 3.18 and Appendix II). It is still relevant to mention that carotenoid content at phyllode tip for DW+N- had no standard error associated since for this parameter it was only possible to analyse one individual associated with this treatment (see 3.2.2).

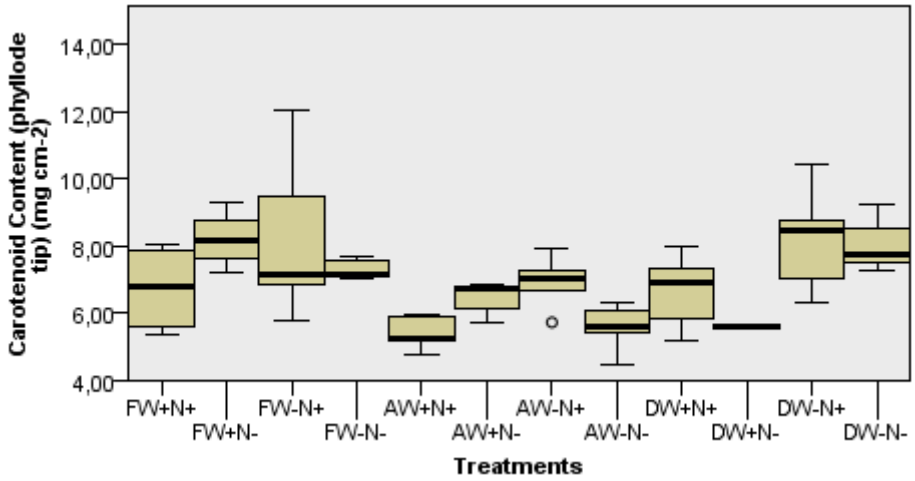


Figure 3.17: Variance analysis box-plot of carotenoid content (phyllode tip) in *A. longifolia*, measured after a growth period of 20 weeks, according to treatment combination (n=55). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution. Symbols represent outliers. Treatment DW+N- has no standard error associated, see text for explanation.

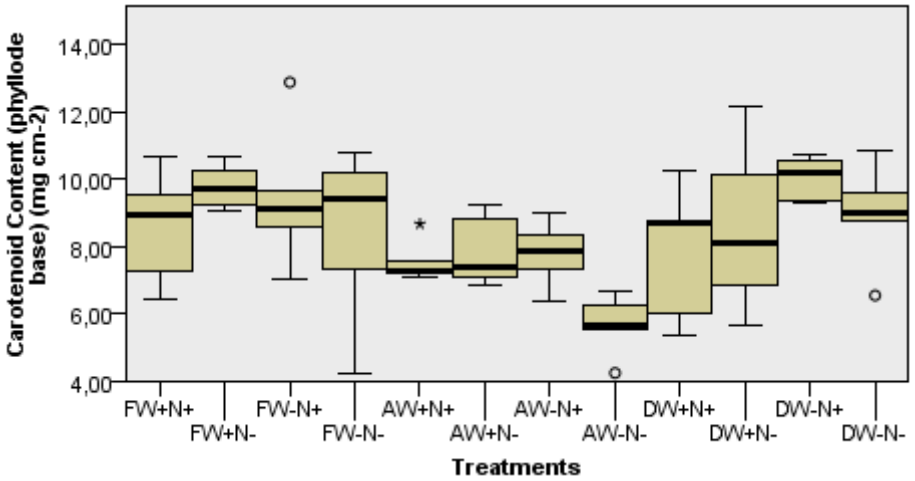


Figure 3.18: Variance analysis box-plot of carotenoid content (phyllode base) in *A. longifolia*, measured after a growth period of 20 weeks, according to treatment combination (n=60). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution. Symbols represent outliers.

3.2.5 Isotopic Analysis

The acacias grown with the treatment combination DW-N- registered a lower mean phyllode $\delta^{15}N$ content with values of -1.5‰, still it is important to note that in all three soils $\delta^{15}N$ content was lower whenever there was no addition of nutrient solution. Phyllode $\delta^{13}C$ content ranged between -32 and -31‰, with lowest mean values being registered in the treatment DW+N-, with average of -32.6 (Table 3.2). In spite the of the Kruskall-Wallis test finding significance regarding phyllode $\delta^{15}N$ content, post-hoc comparisons indicated that there were no significant differences between the treatments (Fig. 3.19 and Appendix II). The phyllode nitrogen (N) and carbon (C) content was on average higher with the treatment combination DW+N-, with values of 3.3% and 44.6%, despite all treatment combinations having similar N content, of about 3%. Additionally, C/N ratio registered higher values with the treatment combination FW-N+, on average 14.4 (Table 3.2).

Table 3.2: Phyllode $\delta^{15}N$, $\delta^{13}C$, nitrogen (N) and carbon (C) content (mean ± SE) according to treatment combinations. F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

Treatments		**Phyllodes**				
		$\delta^{15}N$	**$\delta^{13}C$**	**N%**	**C%**	**CN Ratio**
FW+N+	Mean	4.03	-31.90	3.54	43.85	12.66
	SE	4.32	0.17	0.65	0.66	2.35
FW+N-	Mean	1.03	-32.10	3.68	43.84	12.37
	SE	2.42	0.53	0.82	0.29	3.11
FW-N+	Mean	7.70	-31.60	3.02	43.64	14.47
	SE	4.70	0.72	0.11	1.02	0.77
FW-N-	Mean	2.90	-31.93	3.29	44.07	13.42
	SE	4.77	0.38	0.15	0.71	0.55
AW+N+	Mean	8.13	-32.47	3.61	44.03	12.27
	SE	0.46	0.93	0.32	0.13	1.11
AW+N-	Mean	2.90	-32.33	3.41	44.08	13.89
	SE	4.59	0.32	1.07	0.03	4.72
AW-N+	Mean	8.77	-31.73	3.38	43.95	13.12
	SE	2.60	1.36	0.37	1.38	1.80
AW-N-	Mean	4.30	-31.27	3.27	44.58	13.84
	SE	3.21	1.16	0.50	0.08	2.25
DW+N+	Mean	5.17	-31.67	3.33	43.53	13.23
	SE	2.48	0.97	0.44	0.36	1.96
DW+N-	Mean	-0.13	-32.63	3.34	44.62	13.49
	SE	0.59	0.49	0.40	0.79	1.72
DW-N+	Mean	1.23	-31.83	3.76	43.63	11.60
	SE	0.80	0.60	0.10	0.45	0.36
DW-N-	Mean	-1.50	-31.93	3.66	43.84	12.29
	SE	0.66	1.01	0.71	0.34	2.38

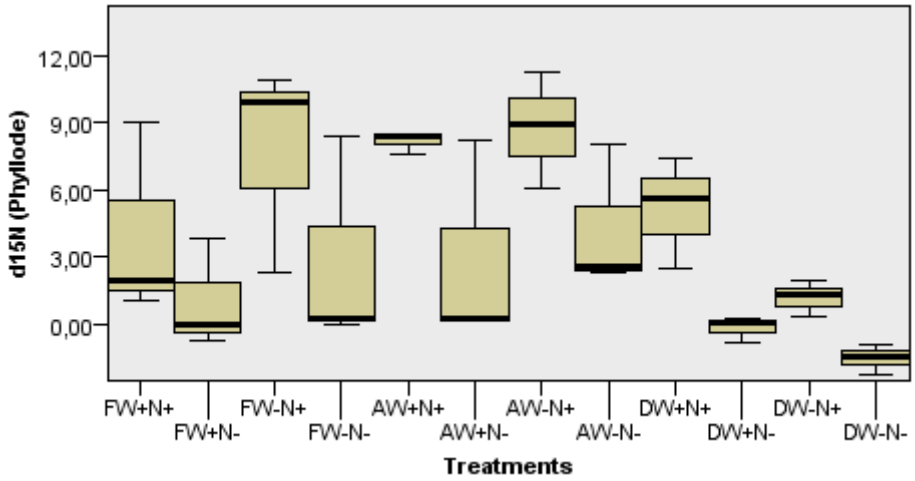


Figure 3.19: Variance analysis box-plot of $\delta^{15}N$ (phyllode) in *A. longifolia*, measured after a growth period of 20 weeks, according to treatment combination (n= 36). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

When considering the same parameters in root nodules, $\delta^{15}N$, $\delta^{13}C$, C% and C/N ratio had lower mean values with the treatment combination FW-N-, with an average of 5.4‰, -53.2‰, 28.2% and 5.9. Whereas nodule N content had the lowest mean values with AW-N+, with 3.7% and, the highest with FW+N+, with 5.6% (Table 3.3). Though, none of these parameters was statistically significant (Appendix II).

Table 3.3: Nodules $\delta^{15}N$, $\delta^{13}C$, nitrogen (N) and carbon (C) content (mean ± SE) according to treatment combinations. F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

Treatments		Nodules				
		$\delta^{15}N$	**$\delta^{13}C$**	**N%**	**C%**	**CN Ratio**
FW+N+	Mean	7.80	-30.93	5.68	40.04	7.13
	SE	1.44	0.35	0.90	1.87	0.82
FW+N-	Mean	7.20	-30.47	5.86	40.93	7.01
	SE	2.91	1.01	0.49	1.21	0.57
FW-N+	Mean	7.97	-30.33	4.97	35.03	7.03
	SE	0.58	0.93	0.43	4.39	0.29
FW-N-	Mean	5.43	-33.27	4.71	28.26	5.59
	SE	5.42	4.97	0.88	19.26	3.47
AW+N+	Mean	9.67	-30.93	4.21	33.00	7.99
	SE	0.60	0.55	1.11	5.65	0.91
AW+N-	Mean	7.73	-30.60	4.84	38.73	8.12
	SE	1.82	0.30	0.79	1.00	1.07
AW-N+	Mean	10.33	-29.67	3.79	38.42	10.31
	SE	1.46	0.68	0.67	0.54	1.54
AW-N-	Mean	6.60	-29.60	5.21	38.11	7.34
	SE	1.61	1.15	0.28	3.02	0.93

DW+N+	Mean	6.87	-30.67	3.64	30.87	8.52
	SE	2.71	1.17	0.96	7.87	0.65
DW+N-	Mean	6.43	-31.77	4.85	37.38	7.80
	SE	1.01	0.42	1.03	5.42	0.74
DW-N+	Mean	7.37	-30.93	5.00	38.60	7.82
	SE	0.45	1.15	0.88	2.85	0.85
DW-N-	Mean	5.67	-31.03	4.78	38.22	8.14
	SE	2.67	0.70	1.18	5.88	0.92

3.3 Photosynthetic pigments analysis

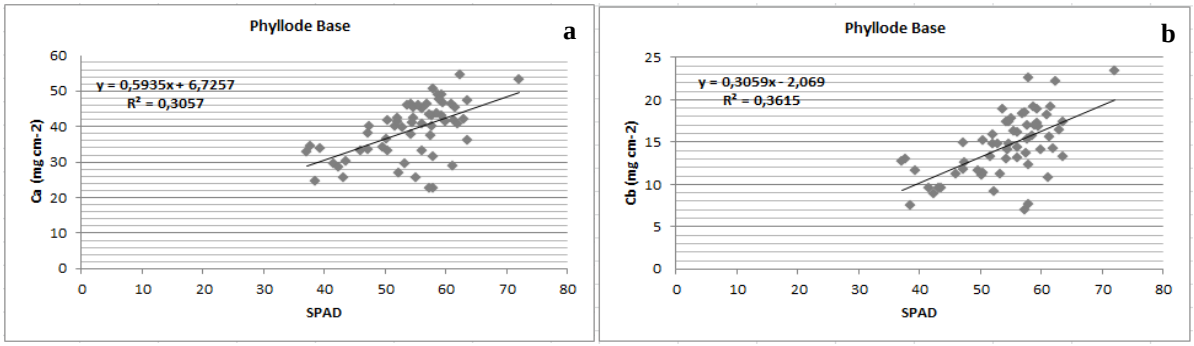
Based on the measurements of photosynthetic pigments concentration and SPAD-502 readings, linear regressions were calculated with the purpose of establishing a calibration model for *A. longifolia* that correlates SPAD data and pigment concentrations.

The resulting linear regressions models (Figure 3.20, Figure 3.21) displayed a very low correlation between pigment concentrations and their corresponding SPAD readings. The predictive capacity of the calibration equations was evaluated by the adjustment coefficient (R^2) and in, both phyllode base and tip, as in all pigments evaluated – C_a; C_b; C_{a+b} and Carotenoids, R^2 value was never above 0.3 (Table 3.4).

Table 3.4: Equations and R^2 values of calibration curves for SPAD-502 readings and photosynthetic pigments content in phyllode base and tip.

	Pigments	Equation	R^2
Phyllode Base	C_a	y=0.5935x+6.7257	0.3057
	C_b	y=0.3059x-2.069	0.3615
	C_{a+b}	y=0.8894x+4.6568	0.3381
	Carotenoids	y=0.1152x+2.2352	0.2197
Phyllode Tip	C_a	y=0.2857x+15.901	0.2617
	C_b	y=0.1368x+3.6514	0.3780
	C_{a+b}	y=0.42255x+19.552	0.3075
	Carotenoids	y=0.052x+4.9031	0.1526

However, the adjustment coefficient values aren't an indicative of the models adequacy, providing solely an estimate of the strength of the correlation. Thus, to evaluate the statistical significance of the models overall F tests were performed (in Appendix IV). All the models, with the exception of the one referring to C_{a+b} (phyllode base), are statistically significant since F critical values were lower than the F values. Therefore, the majority equations obtained allow to predict pigment content based on SPAD-502 measurements. However, for the method to be confirmed further samples are required.



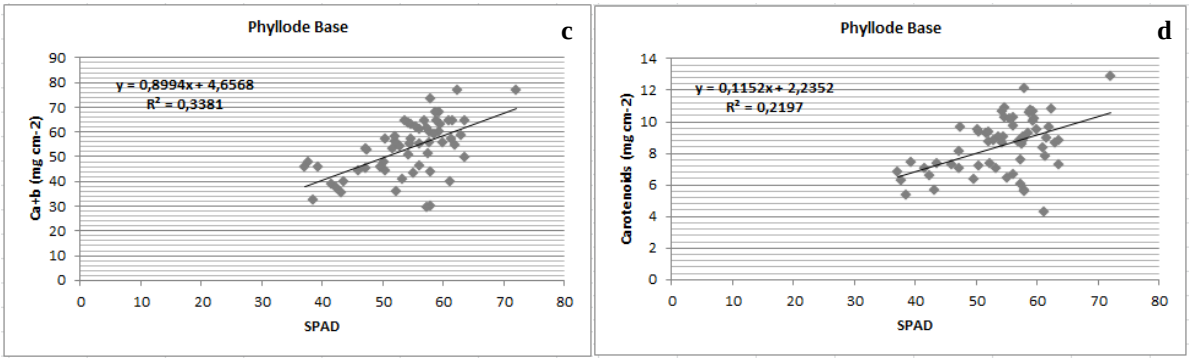


Figure 3. 20: Calibration models relating SPAD-502 readings and (a) chlorophyll a (b) chlorophyll b (c) total chlorophyll and (d) carotenoids content, in phyllode base.

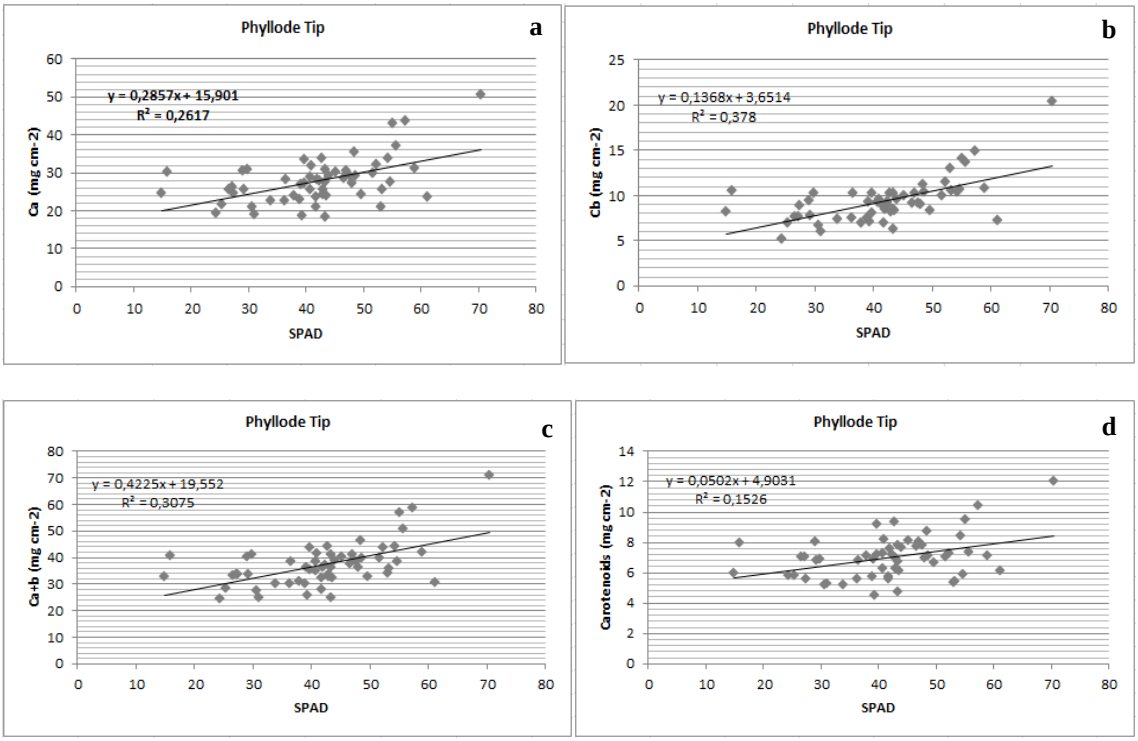


Figure 3.21: Calibration models relating SPAD-502 readings and (a) chlorophyll a (b) chlorophyll b (c) total chlorophyll and (d) carotenoids content, in phyllode tip.

3.4 Bacteria Analysis

A total of 150 colonies were initially obtained after maceration of root nodules, removed from 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. Thus, after purification, a decrease of isolates occurred. A total of 35 isolates were obtained from forest soil nodules, 45 from agricultural soil nodules and 31 from dune soil nodules (Table 3.5). Further analysis showed no evident relationship between the different treatments and the microbial community diversity.

Table 3.5: Number of bacterial isolates found in root nodules in plants of each soil and treatment, before and after purification process.

	Forest				**Agricultural**				**Dune**			
	W+ N+	**W+ N-**	**W- N+**	**W- N-**	**W+ N+**	**W+ N-**	**W- N+**	**W- N-**	**W+N+**	**W+ N-**	**W- N+**	**W- N-**
Before Purification	22	13	3	2	11	8	26	16	13	8	10	18
After Purification	18	12	3	2	10	6	24	5	1	7	8	15

The analysis of the bacterial community present in the nodules was carried out considering the applied treatments, as well as soil type. Still, upon analysis, there was no evident relationship between the different treatments and the microbial community diversity.

As for macroscopic observations, while all colonies observed presented white to yellow tones, in terms of morphology, most isolates could be divided into three major types of colony morphology 1) bands with reticulated edges; 2) viscose bands with smooth edges; 3) small and sparse dots, with slight variations within each group. Microscopic observations determined that most of the isolates consisted of bacillus, almost all endospore forming, except for five isolates from forest soil nodules, which were classified as coccus. Additionally, the Gram staining and biochemical analysis, oxidase, KOH and catalase, tests results were not conclusive.

Regarding molecular analysis, of the three primers initially tested, M13, PH and GTG5, good amplification was only obtained with PH and GTG5. The dendrogram obtained (Fig.3.21) combines the PCR fingerprinting results from both PH and GTG5. Cluster analysis indicated that bacterial isolates grouped in four main clusters and all were distinct, with varying degrees of similarity. This might be because Pearson coefficient considers not only the pattern but also the intensity of the bands of each profile, thus seemingly identical profiles were deemed different by the software. Additionally, the bacterial strains did not cluster according to soil or treatment. Instead, it displayed a heterogeneous distribution, thus indicating that the microbial community might not be conditioned by soil type or treatment.

At last, in order 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, Simpson index (D) of 0.9768 was obtained. As for Shannon-Winer index a score of H'=0.1399.

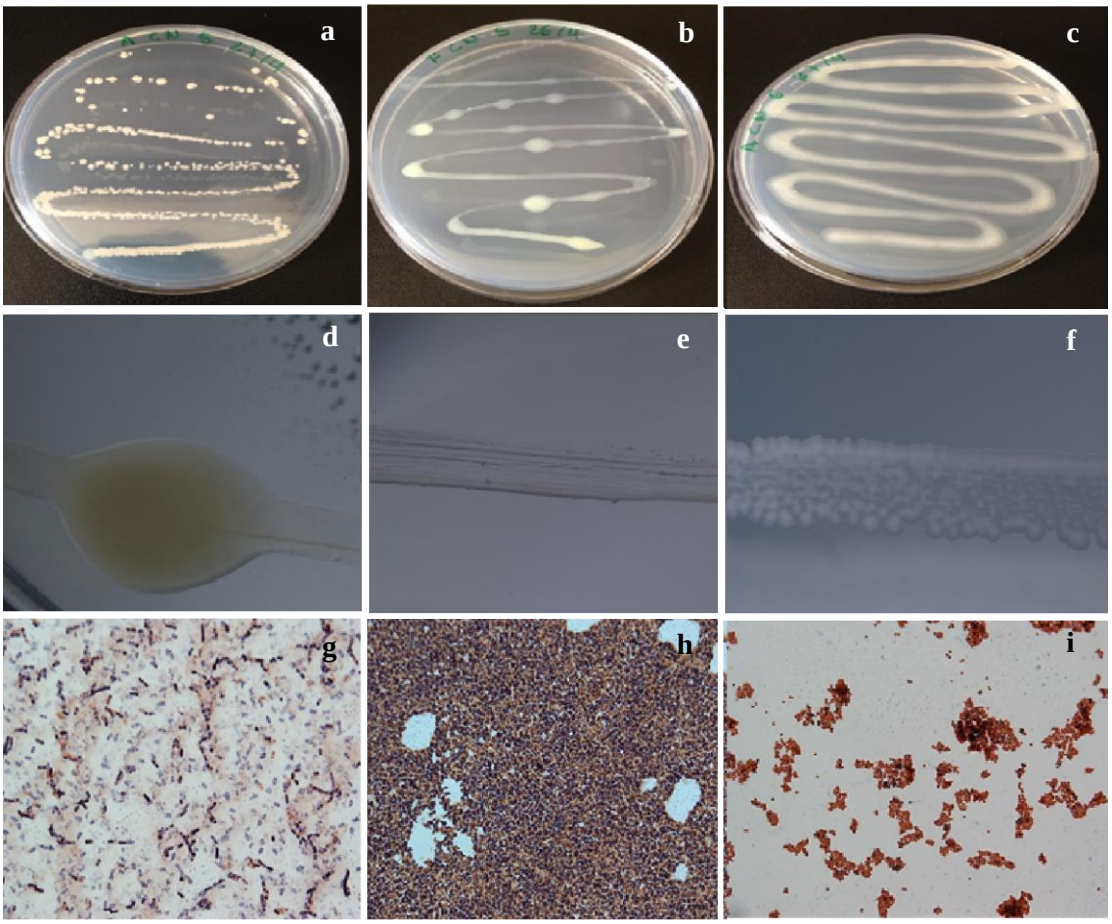


Figure 3.22: Observation of bacteria. (a, b, c) Macroscopic observation of isolated colonies; (a) example of small and sparse dots morphology; (b) example of viscous band with smooth edges morphology; (c) example of band with reticulated edges morphology; (d, e, f) Magnified view of previous colonies; (d) small and sparse dots morphology; (e) viscous band with smooth edges morphology;; (f) band with reticulated edges morphology; (g, h, i) Microscopic observation with 1000x magnification; (g) endospore forming bacillus; (h) non-endospore forming bacillus; (i) coccus.

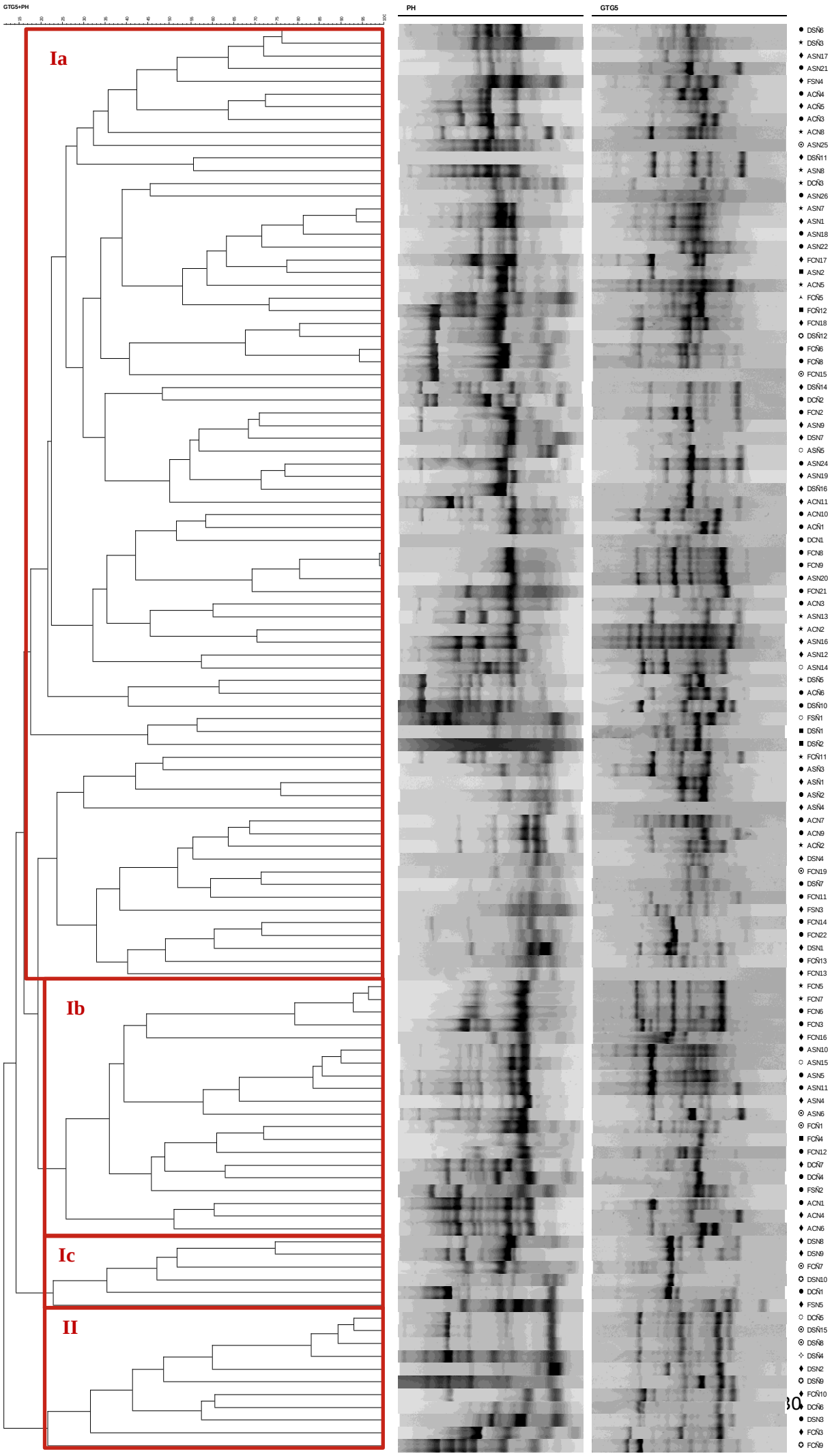


Figure 3.23. Dendogram combining GTG5 and PH fingerprinting results of bacteria isolated form *A. longifolia* root nodules.

4. Discussion

4.1 Do abiotic factors influence *Acacia longifolia* growth and development?

Our study aimed to understand how abiotic factors influenced the growth and development of *A. longifolia*. There is still little information available regarding this topic, since most studies focus on the species impact on the ecosystem, rather than the inverse. Furthermore, most studies differ in the methodology used or in data analysis, rendering it difficult to establish comparisons.

Overall, it was possible to observe a pattern transversal to the analysed parameters. Higher shoot and root increment in length and number of phyllodes were recorded when water was at 70% field capacity and nutrient solution was present (W+N+). A clear decrease was registered with the absence of nutrient solution (W+N-), whereas the values registered for the combinations W-N+ and W-N- had similar distributions or few differences. This pattern was more pronounced in forest and dune soils than in agricultural soil, which had a much more homogeneous distribution of values in the majority of the analysed parameters.

Plant development was mainly affected by soil type, nutrition regime and their interaction. Watering regime had little preponderance in plant development, particularly in forest and agricultural soils (see below). This is not in accordance with findings from other studies, particularly the one conducted by Morais *et al*. (2012), in which *A. longifolia* seedlings were submitted to water stress, which hindered overall plant development. In the study reported here, one particular indicator was the fact that leaf $\delta^{13}C$ content was unaffected by changes in irrigation. This lack of impact may be due to the fact that our greenhouse experiments were conducted during winter, and conditions tested (watering limited to 30% field capacity) were not sufficient to induce water stress in plants.

In what regards the influence of the different nutrition regimes, there was a tendency for acacias grown in the presence of the nutrient solution to have a greater development. This was expected, since a better nutrition is likely to promote growth and promote overall plant fitness. Moreover, it is important to note that in the absence of nutrient solution there was a greater nitrogen fixation, indicated by phyllode $\delta^{15}N$ signature, comparatively, closer to zero. The $\delta^{15}N$ signature is used as an indicator of the nitrogen source used by the plant. This technique of isotope analysis has been previously used in studies concerning *A. longifolia* (Hellman *et al*. 2011; Rascher *et al*. 2012), as well as other nitrogen fixing species, such as *Stauracanthus spectabilis*, *Ulex europaeus* and *Cytisus grandiflorus* (Hellman *et al*. 2011; Rodríguez-Echeverría *et al*. 2009), not only to determine the plant's main source of nitrogen and evaluate the contribution of biological nitrogen fixation, but also as a tool for a better understanding of the nitrogen cycle.

When considering the influence of soil, plants grown in forest and agricultural soil had a greater development, particularly in forest soils with 70% field capacity and nutrient solution (FW+N+), and presented longer shoots and roots, as well as more phyllodes, with a higher hydric content. The results seem to indicate that a higher growth (increment) was accompanied by a greater amount of biomass, particularly in terms of phyllodes and roots. The photosynthetic pigment concentrations were, however, tendentiously lower in forest agricultural soils, which could be explained by the larger phyllode size and higher hydric content, since the cells were more hydrated, and the pigments were more distributed. As for nodules, plants grown in agricultural soils registered a drastically lower number, but with a higher weight, possibly due to the roots being more hydrated. A lower overall development was registered in acacias grown in dune soil, both in terms of increment

and biomass, particularly in the absence of nutrient solution (DW+N-). Additionally, lower phyllode $\delta^{15}N$ content was registered, indicative, as previously mentioned, of higher N_2 fixation.

We hypothesize that these differences in plant development are due to soil characteristics, since the impact of the treatment combinations was different depending on the type of soil. Recent findings indicate the role of soil abiotic factors as a conditioning factor, especially in the early stages of invasion. Acacias notoriously alter soil conditions in the invaded sites, creating favourable conditions which potentiate their development and the establishment of symbiotic relationships with soil bacteria. But firstly they have to overcome soils limiting influence, particularly when soils are poor in nutrients (Le Roux *et al*. 2018). Opposite findings are however referred on a previous study, which found no effect of soil on plant development, including *A. longifolia* (Birnbaum *et al*. 2014). One important difference is that in the mentioned study, the soil of origin did not compose the entirety of the seedlings' growth substrate, while in the present study the acacia seedlings were grown in soil originally collected from the different locations. Several differences exist among the three soil types tested. Forest soil was more acidic, with a higher organic matter content and ammonium-N concentration, since its collection site was a heavily invaded location. Forest soils have been referred as being altered by litter accumulation, with increases in soil organic matter and reduced pH (Rodríguez-Echeverría *et al*. 2009; Souza-Alonso *et al*. 2014). Agricultural soil had the lowest organic matter content, but a higher nutrient concentration, namely nitrate-N and phosphorus, since it was an artificially altered soil, subjected to fertilization. In turn, dune soil had an intermediate composition between forest soil and agricultural soil, both considering organic matter and nutrient availability.

Therefore, we can conclude from the present study that a higher nutrient availability led to a greater plant development, which is in accordance with previous results (Rodríguez-Echeverría *et al*. 2009). This growth, however, wasn't always accompanied by a larger number of nodules contrary to that same study (idem). Our results showed a correlation between more nutrition and greater plant development, accompanied by less nodulation and nitrogen fixation, which is particularly evident in agricultural soil, despite the higher nodule weight registered. It should be stressed that nodulation is a high energy demanding process, and in environments where there is a high nutrient availability, it becomes less or even unnecessary (Stephens and Neyra, 1983). Moreover, it has been reported that the presence of a high NO_3^- content lead to nodule senescence, or even, inhibited its formation (Dupont *et al*. 2012). These may be other conditioning factors that explain the low number of nodules in plants grown in agricultural soil.

In sum, there are several abiotic factors acting simultaneously that influence plant development. The impact of the different treatments will differ depending on which type of soil they are applied. For instance, agricultural soil , which, was richer in nutrients and had higher water retention capacity, was generally little affected by changes in treatment combinations regarding the more prevalent parameters for the assessment of plant development (with the expectation of the number of nodules and phyllode $\delta^{15}N$). This was evident by the more homogeneous distribution of values in the various treatment combinations associated with agricultural soil.

Evidence of this intricate correlation of factors is also indicated by the lower development of acacias grown in dune soil. This soil has a low organic matter and nutrients content, rendering dune soil not only a poor soil, but also a more porous soil, with a lesser capacity for retaining water, and consequently, nutrient solution. Possible indicators of nutrient deficiency were higher root/shoot fresh weight ratio found in the present study. Such an adaptation has been previously identified in other species (Rodríguez-Echeverría *et al*. 2009). Phosphorus deficit has been associated with less phyllode number and a lower leaf area, as well as a lower nodule weight (Leidi *et al*. 2000; Divito *et al*. 2014).

However, in the present study differences in the latter parameter was not statically significant. It is important to mention that effects of phosphorus are still little understood and not always consensual (Divito *et al*. 2014). Though, there might be an indication that plants grown in dune soil we able to surpass the nutrition limitations. While phyllode $\delta^{15}N$ content was lowest in dune soil, the phyllode nitrogen content was similar in acacias from all three soils, that is, despite having different origins, the amount of nitrogen was similar. Thus, acacias in dune soil might have overcome nutrient deficiency, but didn't have a higher growth due to water limitations.

Additionally, our findings showed that, as previously mentioned, with the reduction of nutrient reinforcement, there was a greater fixation of atmospheric nitrogen, since values were closer to zero in the absence of nutrient solution. This trend, though transversal to soil type, was particularly evident in the treatments in dune soil in the absence of nutrient solution, in both watering regimes (DW+N- and DW-N-), in which phyllode $\delta^{15}N$ content was negative.

Thus, our results demonstrate not only the preponderance of abiotic factors such as soil and nutrition in the promotion and hinder of the development of *A. longifolia*, but also that this conditioning is a result of their interaction. That is, abiotic factors have particular effects, for example, nutrition as a growth enhancer and soil as development modulator. However, abiotic factors have to be considered in an integrative perspective, since their influence is not isolated, but act in concert to shape plant development.

4.2 What type of interactions are established between *Acacia longifolia* and nitrogen fixing bacteria?

A total of 111 isolated colonies were obtained in the present study, demonstrating a high diversity of bacteria associated with *A. longifolia* root nodules, highlighting *A. longifolia*'s status as a generalist species (Birnbaum *et al*. 2012; Rodríguez-Echeverría *et al*. 2011). Previous studies indicate, that legumes, such as *A. longifolia*, are mainly nodulated by bacteria from the genera *Bradrhizobium*, particularly *Bradrhizobium japonicum*, *Rhizobium*, *Mesorhizobium* and *Sinorhizobium*, as well as *Ensifer* and *Acinetobacter* (Rodríguez-Echeverría *et al*. 2003; Fterich *et al*. 2012; De Meyer *et al*. 2015; Souza-Alonso *et al*. 2017; Kamutando *et al*. 2019). Although the bacterial community associated with *Acacia* spp. seems to vary, the genus *Bradrhizobium* appears to be shared across most locations, and is in association with several species of acacia. The importance of this genus relates to the fact that this is one of the main rhizobial taxa to express genes associated with plants promoting traits, such as the metabolism of nutrients, vitamins and nitrogen (Kamutando *et al*. 2017, 2019). Therefore, the genus will play an essential role in plant development and in the processes underlying invasiveness success.

This bacterial diversity, associated with *A. longifolia* grown in different soils, can be explained by several reasons. Firstly, the bacteria community in the soil sampling sites might be distinctive or have different abundances of specific bacteria involved in nodulation. A study conducted with *A. dealbata* demonstrates that biogeography and soil characteristics, particularly nutrients content, influence which bacteria associate with Acacia (Kamutando *et al*. 2017). Secondly, some of the isolates might be, on one hand, non-symbiotic endophytic bacteria. The presence of these microorganisms has been reported not only in other *Acacia* species (Fteich *et al*. 2012; Ndlouv *et al*. 2013), but also in other legumes (Senthilkumar *et al*. 2009; Li *et al*. 2008).

Some of the genera of non rhizobial endophytes identified in previous reports (De Meyer *et al*. 2015), in association with root nodules of other legume species, are Gammaproteobacteria, particularly *Enterobacter* and *Pseudomonas*, Ochrobactrum and Fimobacteria, particularly, *Bacillus* and *Staphiloccocus*. One the other hand, there might be the result of horizontal transfer of rhizobial nodulating genes between bacteria associated with *Acacia* and bacteria already present in the soil (Rodríguez-Echeverría *et al*. 2003, 2010; Rout *et al*., 2012; Ndlouv *et al*. 2013) and so increasing the number of bacteria associated with the plant. The genes, responsible for nitrogen fixation and nodulation, particularly, *nif*D and *nod*A genes, are present in symbiotic plasmids or islands. These can be laterally transferred to bacteria present in the soil, conferring them the capacity to not only to form and enter nodules, but also to biologically fix nitrogen (idem).

The total number of isolates reported here is a seemingly higher number compared to similar studies. In previous reports, concerning *A. longifolia*, 38 and 44 isolates were obtained from root nodules collected in the São Jacinto Natural Reserve in two separate occasions (Rodríguez-Echeverría *et al*. 2010, 2007), while a study conducted in their native Australia, obtained 60 isolates from *A. longifolia* root nodules. Though from a different plant species, a similar study, also found 112 isolates in *A. saligna* in Portugal, a number closer to our findings, while in Tunisia 50 strains were isolated from *A. tortilis* (Fterich *et al*. 2012). However it's difficult to establish true comparisons. Studies concerning plant-rhizobia interactions have frequently different methodological approaches and analysis of results (Fteich *et al*. 2012; Ndlouv *et al*. 2013; Crisóstomo *et al*. 2013; Rodríguez-Echeverría *et al*. 2007, 2009), which might condition and justify differences obtained.

Furthermore, the isolates were genetically diverse and distinguished. Although the strains were not identified, polymorphisms resulting from amplification with two different universal primers, GTG5 and PH allowed a clear separation of the bacterial strains. These primers are characterized by being universal primers, complementary to conserved and repetitive sequences throughout the genome, which translates into a great discriminatory capacity, facilitating the distinction between intraspecific bacteria (Soqui, 2007; Chaves, 2005; Ramírez-Castrillho *et al*. 2014). GTG5 has been successfully used in previous studies of rhizobia and non-rhizobia endophytes in other leguminous species (De Meyer *et al*. 2015). Based on the patterns of both primers, cluster analysis was carried out with Pearson coefficient. Later Shannon-Wiener and Simpson coefficients were determined, with a cut-off of 50%.

In our present study, clustering analysis showed that strains from the same soil and treatment combinations grouped into different clusters, while similar microorganisms were present in different soil types. Similar results were obtained for studies conducted with wild and crop legumes, in Italy (Moschetti *et al*. 2005). These data seem indicative not only of the presence of a varied and heterogeneous bacterial community, but also of the absence of relationship between the isolated strains and soil origin, again indicating a diverse community in each site. However, a previous report (Palmer *et al*. 2000) indicates an influence of soil, in particular the combination of soil factors, on rhizobial diversity.

Further studies are required, in particular the identification of the isolated strains, to better understand and characterize the rhizobial community associated with the different soils, and to have a more concrete notion of the diversity associated with *A. longifolia* . Since, acacia performance and fitness are influenced by rhizobia diversity, but different bacterial stains might vary in symbiotic effectiveness (Keet *et al*. 2017). Therefore, is essential to determine which bacteria establish mutualistic interactions with acacia.

Additionally, it was not possible to establish any relationship between isolates and the parameters applied during the experimental period. That is, there wasn't an evident correlation between the number of isolates obtained and the different treatments, which indicated that the applied parameters didn't influenced or conditioned the number of bacteria nodulating *A. longifolia*. Furthermore, the different amount of bacteria isolated from acacias grown under different conditions didn't seem to, positively or negatively, influence plant development, but then again the determination of bacteria strains would allow a more clear understanding of the presence, or absence of any correlation.

4.3 Can SPAD be correlated with photosynthetic pigment quantification?

The quantification of photosynthetic pigments is time a consuming and destructive process, and so we attempted to find a calibration equation correlating SPAD-502 readings and photosynthetic pigments content for *A. longifolia* phyllodes. Our results showed low adjustment coefficients (R^2) varied between 0.1 and 0.3; however R^2 values don't determine the adequacy of the model. Despite these low coefficients, the relationship between spectrophotometric chlorophyll and carotenoids measurements and SPAD-502 readings was statistically significant Thus, the calibration equations have predictive capacity, however it's necessary to analyse more samples to validate the model. The low adjustment coefficients may result from the fact that SPAD measurements are affected by several factors such as leaf thickness and water content (Marenco *et al*. 2009). Furthermore, there is non-uniform distribution of chloroplasts in the leaf, influenced by different light conditions (Xiong *et al*. 2015). Moreover, throughout the phyllodes, the vascular system (xylem and phloem) was present in two main longitudinal areas of vascularization, and it was difficult to remove disks without these tissues.

A correlation between SPAD-502 readings and photosynthetic pigments content has been established for an assortment of species. A previous report was able to establish correlation ($R^2 > 0.9$) for *Arabidopsis thaliana* (Ling *et al*. 2011), thus facilitating the determination of photosynthetic pigments for this species. Additionally, a calibration curve was also determined for birch, wheat and potato (Uddling *et al*. 2007). However, the correlation found for potato was relatively weak ($R^2 \approx 0.5$), illustrates that this correlation is not only species-specific but also that, its success might not be the same for every specie.

This evaluation has not been done before, as far as we know, for other *Acacia* spp., including *A. longifolia* and would allow a much faster and less laborious quantification of photosynthetic pigments.

5. Conclusion and Future Perspectives

Acacia longifolia is an aggressive invader, disrupting habitats and communities both in Portugal and in other regions around the world. Understanding what conditions its expansion and influences its development is of paramount importance. To achieve that, we need acquire more information, not only to better understand the complex process underlying its invasive success but to encounter ways to avoid its establishment and control its dispersal.

Overall, our findings showed that acacias development is modulated by abiotic factors. Through the greenhouse trials and subsequent analysis, we found evidence that nutrition and soil conditions shape plant fitness, with soil conditioning development and nutrition promoting growth. Additionally, we highlighted the importance of biological nitrogen fixation, particularly in situations of nutrient scarcity.

Furthermore, our results regarding rhizobia analysis indicated the high bacterial diversity associated with *A. longifolia*. Though, additional studies are necessary in order to identify the isolated bacteria, as well as apply different technologies, such NGS (next generation sequencing), in order to have a better and more complete understanding of the rhizobial community associated with *A. longifolia* and how it might influence development and plant fitness.

Lastly, further studies are required, following an integrative perspective, since *A. longifolia* success as prolific invasive species results of the conjugation and interaction of several factors. Standardized methodologies should also be defined to facilitate comparisons between different studies.

6. References

Antunes, C., Pereira, A. J., Fernandes, P., Ramos, M., Ascensão, L., Correia, O. (2018). Understanding plant drought resistance in a Mediterranean coastal sand dune ecosystem: differences between native and exotic invasive species. *Journal Plant Ecology*, **11** (1): 26-38.

Bartholomew, J. W., Mittwer, T. (1952). The Gram stain. *Bacteriology Reviews*, **16** (1): 1-29

Birnbaum, C., Barrett, L. G., Thrall, P. H., & Leishman, M. R. (2012). Mutualisms are not constraining cross-continental invasion success of Acacia species within Australia. *Diversity and Distributions* , **18**(10).

Birnbaum, C., Bissett, A., Thrall, P. H., & Leishman, M. R. (2014). Invasive legumes encounter similar soil fungal communities in their non-native and nitive ranges in Australia. *Soil Biology & Biochemistry*, **76**:210-217.

Brockwell , J., Searle, S. D., Jeavons, A. C., & Waayers, M. (2005). *Nitrogen Fixation in Acacias: as Untapped Resource for Sustainable Plantations, Farm Forestry and Land Reclamation.* ACIAR Monograph No. 115, 132p (2005).

Chaves, S. (2005). "Diversidade de procariotas sulfo-redutores e desnitrificantes em amostras ambientais". *Tese de Douturamento*, Capítulo 4: Diversidade de SRP em amostras ambientais, pp. 86-87.

Correia, M., Castro, S., & Rodríguez-Echeverría, S. (2015). Reproductive success of *Acacia longifolia* (Fabaceae, Mimosoideae). *Australian Journal of Bothany*, **63**:387-391.

Correia, M., Montesinos, D., French, K., & Rodríguez-Echeverría, S. (2016). Evidence for enemy release and increased seed production and size for two invasive Australian acacias. *Journal of Ecology*, **104**:1391-1399.

Crisóstomo, J. A., Rodríguez-Echeverría, S., & Freitas, H. (2013). Co-introduction of exotic rhizobia to the rhizosphere of the invasive legume *Acacia saligna*, an intercontinental study. *Applied Soil Ecology*, **64**:118-126.

Dawson, T. E., Mambelli, S., Plamboeck, A. H., Templer, H. P., & Tu, K. P. (2002). Stables Isotopes in Plant Ecology. *Annual Review of Ecology, Evolution and Systematics*, **33**:507- 59.

De Meyer, S. E., De Beuf, K., Vekeman, B., & Willems, A. (2015). A large diversity of non-rhizobial endophytes dound in legume root nodules in Flanders (Belgium). *Soil Biology & Biochemistry*, 1 - 11.

Divito, G. A., & Sadras, V. O. (2014). How do phosphorus, potassium and sulphur affect plant growth and biological nitrogen fixation in crrop and pasture legumes? A meta-analysis. *Field Crops Research* , **156**:161-171.

Duarte, L. N. (2016). *"Plantas Invasoras no Sul de Portugal - Uma Abordagem Biogeográfica"*. Tese de Mestrado em Biologia da Conservação, Universidade de Évora.

Dupont, L., Alloing, G., Pierre, O., Msehli, S. E., Hopkins, J., Hérouart, D. (2012). *The Legume Root Nodule: From Symbiotic Nitrogen Fixation to Senescence* . Senescence, Dr. Tetsuji Nagata (Ed.), In Tech.

Fterich, A., Mahdhi, M., Lafuente, A., Pajuelo, E., Caviedes, M. A., Rodriguez-Llorente, I. D. (2012). Taxonomic and symbiotic diversity of bacteria isolated from nodules of *Acacia tortilis* subsp. *raddiana* in arid soils of Tunisia. *Canadian Journal of Microbiology*, **58**: 738-751.

Hellmann, C., Sutter, R., Rascher, K. G., Máguas, C., Correia, O., & Werner, C. (2011). Impact of an exotic N_2-fixing Acacia on composition and N status of a native Mediterranean community. *Acta Oecologica*, **37**: 43-50.

Hungria, M., O'Hara, G. W., Zilli, J. E., Araujo, R. S., Deaker, R., & Howieson, J. G. (2016). Isolation and growth of rhizobia. In *Working with Rhizobia, Chapter 3* (pp. 39-41). Australian Centre for International Agricultural Research Canberra : In J. G. Howieson and M.J Dilworth (Eds.).

Kamutando, C. N., Vikram, S., Kamgan-Nkuekam, G., Makhalanyane, T. P., Greeve, M., Le Roux, J. J. (2017). Soil nutritional status and biogeography influence rhizosphere microbial communities associated with the invasive tree Acacia dealbata. *Scientific Reports*.

Kamutando, C. N., Vikram, S., Kangan-Nkuekam, G., Makhalanyane, T. P., Greeve, M., Le Roux, J. J. (2019). The Functional Potential of the Rhizospheric Microbiome of an Invasive Tree Species, *Acacia dealbata*. *Microbial Ecology*, **77**:191-200.

Keet, J., Ellis, A. G., Hui, C., & Le Roux, J. J. (2017). Legume -rhizobium symbiotic promiscuity and effectiveness do not affect plant invasiveness. *Annals of Botany*, 119:1319-1331.

Le Maitre, D. C., Gaertner, M., Marchante, E., Ens, E., Holmes, P. M., Pauchard, A.(2011). Impacts of invasive Australian acacias: implications for management and restoration. *Diversity Distrib.*, **17**:1015-1029.

Le Roux, J. J., Ellis, A. G., Zyl, L., Hosking, N. D., Keet, J., & Yannelli, F. A. (2018). Imporrtance of soil legacy effects and successful mutualistic interactions during Australian acacia invasions in nutrient-poor environments. *Journal of Ecology*, 106:2071-2081.

Leidi, E. O., & Rodríguez-Navarro, D. N. (2000). Nitrogen and phosphorus availability limit N_2 fixation in bean. *New Phytologist*, **147**:337-346.

Li, J. H., Wang, E. T., Chen, W. F., & Chen, W. X. (2008). Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. *Soil Biology and Biochemestry*, **40**(1):238-246.

Lichtenthaler, H. (1987). Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. London: Academic Press.

Ling, Q., Huang, W., & Jarvis, P. (2011). Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. *Photosynthesis Research* , **107**(2): 209-214.

Madigan, M. T, Martinko, J. M, Bender, K. S, Buckley, D. H, & Stahl, D. A. (2017) *Brock, Biology of microorganisms* (Fifteenth edition). Boston: Pearson.

Marchante, E., Kjoller, A., Struwe, S., & Freitas, H. (2008). Short- and long-term impacts of Acacia longifolia invasion the belowground processes of a Mediterranean coastal dune ecosystem. *Applied Soil Ecology*, **40**: 210-217.

Marchante, H., Freitas, H., & Hoffman, J. H. (2011). Post-clearing recovery of coastal dunes invaded by Acacia longifolia: is duration of invasion relevant for manegement success? *Journal of Applied Ecology*, **48**:1295-1304.

Marchante, H., Marchante, E., Freitas, H., & Hoffman, J. H. (2015). Temporal changes in the impacts on plant communities of an invasive alien tree, Acacia longifolia. *Plant Ecology*, **216**: 1481-1498.

Marenco, R., Antezana-Vera, S., & Nascimento, H. (2009). Relationship between specific leaf area, leaf thickness, leaf water content and SPAD-502 readings in six Amazonian tree species. *Photosynthetica*, **47**:184-190.

Morais, M. C., & Freitas, H. (2012). The acclimation potential of *Acacia longifolia* to water stress: Implications for invasivness. *Plant Science* , **196**:77-84.

Moschetti, G., Peluso, A., Protopapa, A., Anastasio, M., Pepe, O., & Defez, R. (2015). Use of nodulation pattern stress tolerance nodC gene amplification, RAPD-PCR and RFLP-16S rDNA analysis to discriminate genotypes of *Rhizobium leguminosarum* biovar *viciae*. *Systematic and Applied Microbiology*, **28**:619-631.

Ndlovu, J., Richardson, M. D., Wilson, J., & Le Roux, J. J. (2013). Co-invasion of South African ecosystems by an Australian leguma and its rhizobial symbionts. *Journal of Biogeography*, **40**:1240-1251.

Palmer, K. M., & Young, J. P. (2000). Higher Diversity of *Rhizobium leguminosarum* Biovar *viciae* Populations in Arable Soils than in Grass Soils. *Applied and Environmental Microbiology*, **66**(6):2445-2450.

Ramírez-Castrillón, M., Mendes, S. D., Inostroza-Ponta, M., & Valente, P. (2014). (GTG)5 MSP-PCR Fingerprinting as a Technique for Driscrimination of Wine Associated Yeasts? *PLoS ONE*, **9**(8):e105870.

Rascher, K., Werner, C., Máguas, C., & Correia, O. (2009). Tracing seasonal changes in water use of an invasive Acacia and a native pine in southern Portugal by measurement of sap flow. *Acta Horticulturae*, **846**:209-216.

Rascher, K. G., Groβe-Stoltenberg, A., Máguas, C., & Werner, C. (2011a). Understory Invasion by *Acacia longifolia* Alters the Water Balence and Carbon Gain of a Mediterranean Pine Forest. *Ecosystems*, **14**: 904-919.

Rascher, K., Groβe-Stoltenberg, A., Máguas, C., Meira-Neto, J., & Werner, C. (2011b). *Acacia longifolia* invasion impacts vegetation structure and regeneration dynamics in open dunes and pine forests. *Biological Invasions* , **13** (5): 1099-1113.

Rascher, K., Hellman, C., Máguas, C., & Werner, C. (2012). Community scale ^{15}N isoscapes: Tracing the spatial impact of an exotic N2-fixing. *Ecology Letters*, **15**(5):484-491.

Richardson, D. M., Peysek, P., Rejmánek, M., Barbour, M. G., Panetta, D., & West, C. J. (2000). Naturalazation and invasion of alien plants: concepts and definitions. *Diversity and Distributions*, **6**:93-107.

Richardson, D. M., & Rejmánek, M. (2011). Trees and shrubs as invasive alien species - a global review. *Diversity and Distributions*, **17**:788-809.

Rodríguez-Echeverría, S., Pérez-Fernández, M. A., Vlaar, S., & Finnan, T. (2003). Analysis of legume-rhizobia symbiosis in shrubs from central western Spain. *Journal of Applied Microbiology*, **95**:1367-1374.

Rodríguez-Echeverría, S., Crisóstomo, J. A., & Freitas, H. (2007). Genetic Diversity of Rhizobia Associated with *Acacia longifolia* in Two Stages of Invasion of Coastal Sand Dunes. *Applied and Environmental Microbiology*, **73** (15): 5066-5070.

Rodríguez-Echeverría, S., Crisóstomo, J. A., Nabais, C., & Freitas, H. (2009). Belowground mutualists and the invasive ability of *Acacia longifolia* in coastal dunes of Portugal. *Biological Invasions*.

Rodríguez-Echeverría, S. (2010). Rhizobial hitchhickers from Down Under: invasional meltsown in a plant-bacteria mutualism? *Journal of Biogeography*, **37**:1611-1622.

Rodríguez-Echeverría, S., Le Roux, J. J., Crisóstomo, J. A., & Ndlovu, J. (2011). Jack-of-all-trades and master of many? How does asscoiated rhizobial diversity influence the colonization success of Australian Acacia species? *Diversity and Distributions*, **17**:946-957.

Rout, M. E., & Callaway, R. M. (2012). Interactions between exotic invasive plants and soil microbes in the rizosphere suggest that "everything is not everywhere". *Annals of Bothany* , **110**: 213-222.

Sadava, D., Hills, D., Heller, C., & Berenbaum, M. (2014). *Life: The Science of Biology, Tenth Edition.* Sinauer Associates, Inc., pp748.

Senthilkumar, M., Swarnalakshmi, K., Govindasamy, V., Lee, Y. K., & Annapurna, K. (2009). Biocontrol potential of soybean bacterial endophytes against charcol rot fungus, *Rhizoctonia bataticola*. *Current Microbiology*, **58**:288-293.

Soqui, M. V. (2007). "Carcterização fenótipica e molecular de isolados de *Campylobacter jejuni* e *Campylobacter coli* de origem avícola". *Tese Douturamento*, PARTE III: Caracterização molecular, Capítulo 3: Análise por PCR-fingerprinting, pp 142-143.

Souza-Alonso, P., Guisande-Collazo, A., & González, L. (2014). Gradualism in *Acacia dealbata* Link invasion: Impact on soil chemestry and microbial community over a chronological sequence. *Soil Biology & Biohemestry*, **80**:315-323.

Souza-Alonso, P., Rodríguez, J., González, L., & Lorenzo, P. (2017). Here to stay: Recent advances and perspectives about Acacia invasion in Mediterranean areas. *Annals of Forest Science* , **74**: 55.

Stephens, B. D., & Neyra, C. A. (1983). Nitrate and nitrite reduction in relation to nitrogenase ctivity in soybean nodules and *Rhizobium japonicum* bacteroids. *Plant Physiology*, **71**:731-735.

Uddling, J., Gelang-Alfredsson, J., Piikki, K., & Pleijel, H. (2007). Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. *Photosynthesis Research*, **91**(1): 37-46.

Ulm, F., Hellman, C., Cruz, C., & Máguas, C. (2017). N/P imbalance as a key driver for the invasion of oligotrophic dune systems by a woody legume. *Oikos*, **126**: 232-240.

Vicente, S. (2016). *"Assessement of genetic variability in the exotic invasive species Acacia longifolia using molecular markers"*. Tese de Mestrado, Faculdade de Ciências, Universidade de Lisboa.

Xiong, D., Chen, J., Yu, T., Gao, W., Ling, X., Li, Y.,(2015). SPAD-based leaf nitrogen estimation is impacted by environmental factors and crop leaf characteristics. *Scientific Reports*, **5**:13389.

6. Appendices

6.1 Appendix I- Formulations

Table 6.1: Modified Hoagland Solution with final concentrations.

Nutrients	Concentration
Potassium nitrate KNO_3	1.5 mM
Calcium nitrate $Ca(NO_3)_2$	1mM
Mono ammonium phosphate $NH_4H_2PO_4$	0.5 mM
Magnesium sulphate $MgSO_4$	0.25 mM
Potassium chloride KCl	50 µM
Boric acid H_3BO_3	25 µM
Manganese sulphate $MnSO_4.H_2O$	2 µM
Zinc sulphate $ZnSO_4.7H_2O$	2 µM
Copper Sulphate $CuSO.5H_2O$	0.5 µM
Ammonium molybdate $(NH_4)6Mo7O_{24}$	0.5 µM
FeNaEDTA	2 µM

Table 6.2: Yeast Mannitol Medium

Component	**Concentration (g/L)**
Mannitol	10.0
K_2HPO_4	0.5
$MgSO_4.7H_20$	0.2
NaCl	0.1
Yeast Extract	0.5
Agar	15
Distilled water to complete 1L pH adjusted to 6.8	

6.2 Appendix II- Significance Levels

Table 6.3: Kruskall-Wallis hypothesis test summary, according to treatment combinations ($p \leq 0.05$).

Null hypothesis	Significance	Decision
The distribution of Shoot Increment is the same among the Treatments categories	0.004	Reject the null hypothesis
The distribution of Root Length is the same among the Treatments categories	0.036	Reject the null hypothesis
The distribution of Root/Shoot Length Ratio is the same among the Treatments categories	0.129	Retain the null hypothesis
The distribution of Root/Shoot Fresh Weight Ratio is the same among the Treatments categories	0.002	Reject the null hypotheis
The distribution of Root Hydric Content is the same among the Treatments categories	0.000	Reject the null hypothesis
The distribution of Number of Phyllodes is the same among the Treatments categories	0.000	Reject the null hypothesis
The distribution of Phyllode Hydric Content is the same among the Treatments categories	0.000	Reject the null hypothesis
The distribution of Number of Nodules is the same among the Treatments categories	0.001	Reject the null hypothesis
The distribution of Nodule Weight is the same among the Treatments categories	0.066	Retain the null hypothesis
The distribution of Nodule Weight/Number Ratio is the same among the Treatments categories	0.042	Reject the null hypothesis
The distribution of Ca+b Content (phyllode tip) is the same among the Treatments categories	0.079	Retain the null hypothesis
The distribution of Ca+b Content (phyllode base) is the same among the Treatments categories	0.356	Retain the null hypothesis
The distribution of Carotenoid Content (phyllode tip) is the same among the Treatments categories	0.002	Reject the null hypothesis
The distribution of Carotenoid Content (phyllode base) is the same among the Treatments categories	0.011	Retain the null hypothesis
The distribution of $\delta^{15}N$ (phyllode) is the same among the Treatments categories	0.014	Reject the null hypothesis
The distribution of $\delta^{13}C$ (phyllode) is the same among the Treatments categories	0.741	Retain the null hypothesis
The distribution of N% (phyllode) is the same among the Treatments categories	0.695	Retain the null hypothesis
The distribution of C% (phyllode) is the same among the Treatments	0.453	Retain the null hypothesis

(continuation of table 6.3)

categories		
Null hypothesis	**Significance**	**Decision**
The distribution of CN Ratio is the same among the Treatments categories	0.745	Retain the null hypothesis
The distribution of $\delta^{15}N$ (nodule) is the same among the Treatments categories	0.240	Retain the null hypothesis
The distribution of $\delta^{13}C$ (nodule) is the same among the Treatments categories	0.267	Retain the null hypothesis
The distribution of N% (nodule) is the same among the Treatments categories	0.177	Retain the null hypothesis
The distribution of C% (nodule) is the same among the Treatments categories	0.235	Retain the null hypothesis
The distribution of CN Ratio (nodule) is the same among the Treatments categories	0.153	Retain the null hypothesis

Table 6.4: Pairwise comparisons summary of statistical significant variables, according to treatment combinations ($p \leq 0.05$). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

Treatments		
Variable	**Categories**	**Adjusted Significance**
Shoot Increment	DW+N- FW+N+	0.049
Root/Shoot Fresh Weight	FW+N+ DW+N-	0.010
	FW+N+ AW-N+	0.014
Number of Phyllodes	DW+N- AW+N+	0.028
	DW+N- DW-N+	0.010
	DW+N- FW+N+	0.000
	DW-N- FW+N+	0.004
	FW+N- FW+N+	0.007
Number of Nodules	AW+N+ FW+N+	0.021
	AW-N+ FW+N+	0.045
Carotenoid Content (phyllode tip)	AW+N+ FW+N-	0.034
Carotenoid Content (phyllode tip)	AW-N- FW+N-	0.021
	AW-N- DW-N+	0.009
Phyllodes Hydric Content	DW+N- AW-N-	0.041
	DW+N- AW-N+	0.016
	DW+N- DW-N+	0.012
	DW+N- FW+N+	0.000
	DW+N- AW+N+	0.000
	DW-N- FW+N+	0.042
	DW-N- AW+N+	0.035
Roots Hydric Content	DW+N- AW-N-	0.029
	DW+N- AW-N+	0.019
	DW+N- AW+N+	0.005

6.3 Appendix III- Values for absolute chlorophyll content in *Acacia longifolia* phyllode

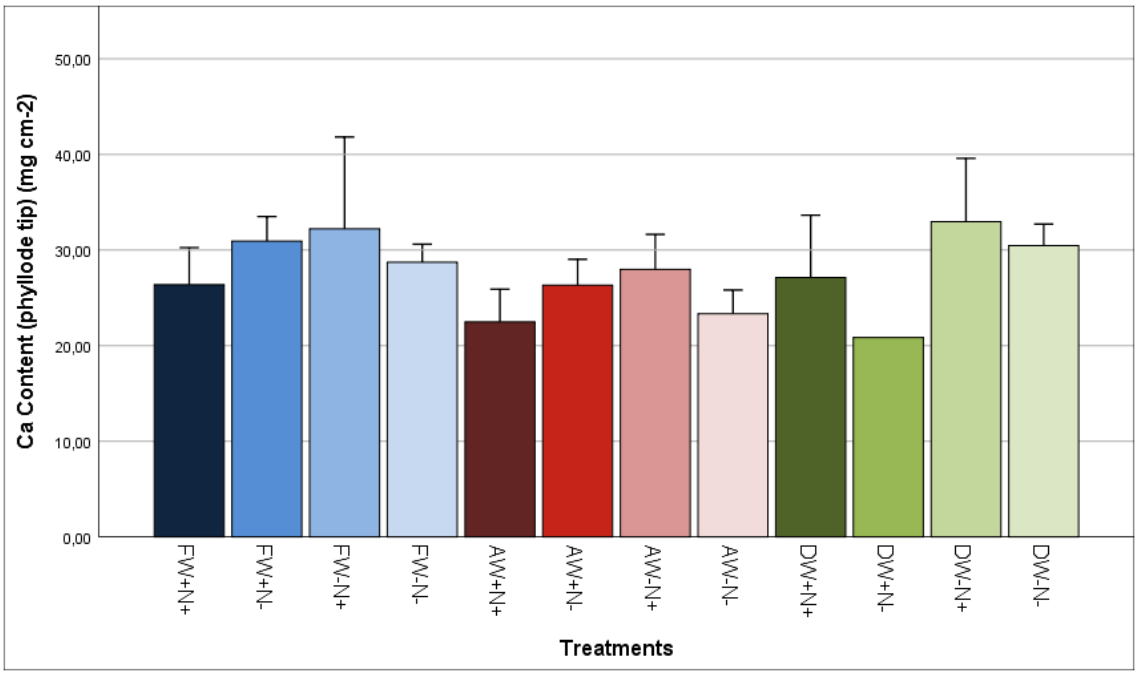


Figure 6.1: Average distribution of C_a content (phyllode tip) (mean ± 2SE) of *A. longifolia*, according to treatment combination (n= 55). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

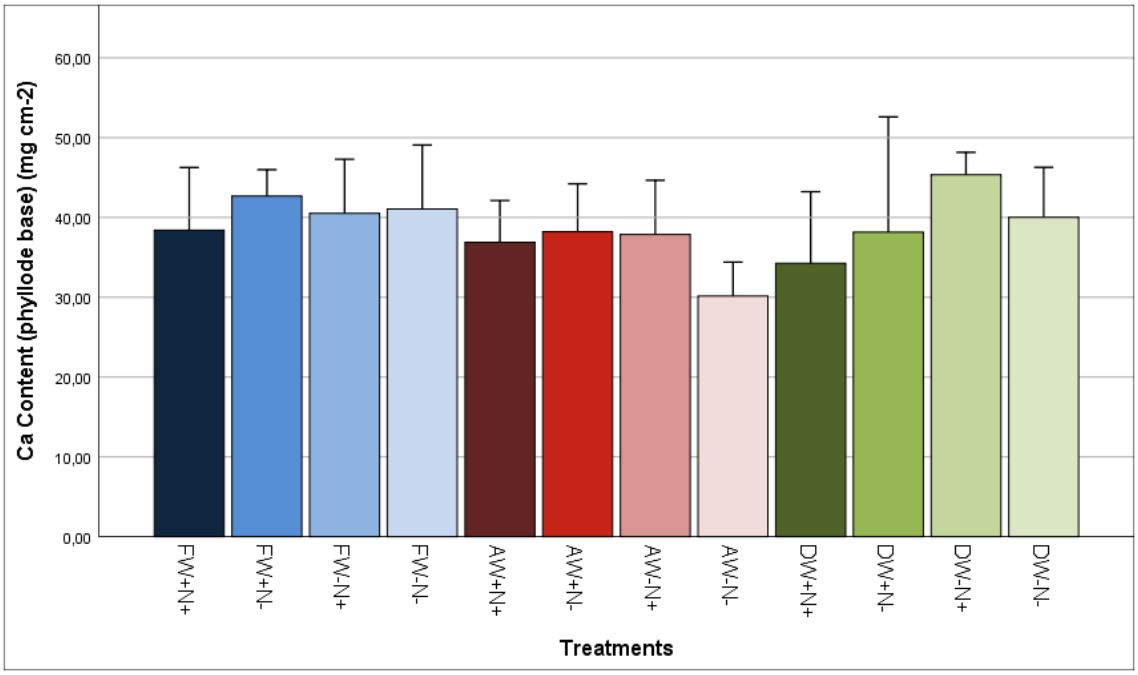


Figure 6.2: Average distribution of C_a content (phyllode nase) (mean ± 2SE) of *A. longifolia*, according to treatment combination (n= 60). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

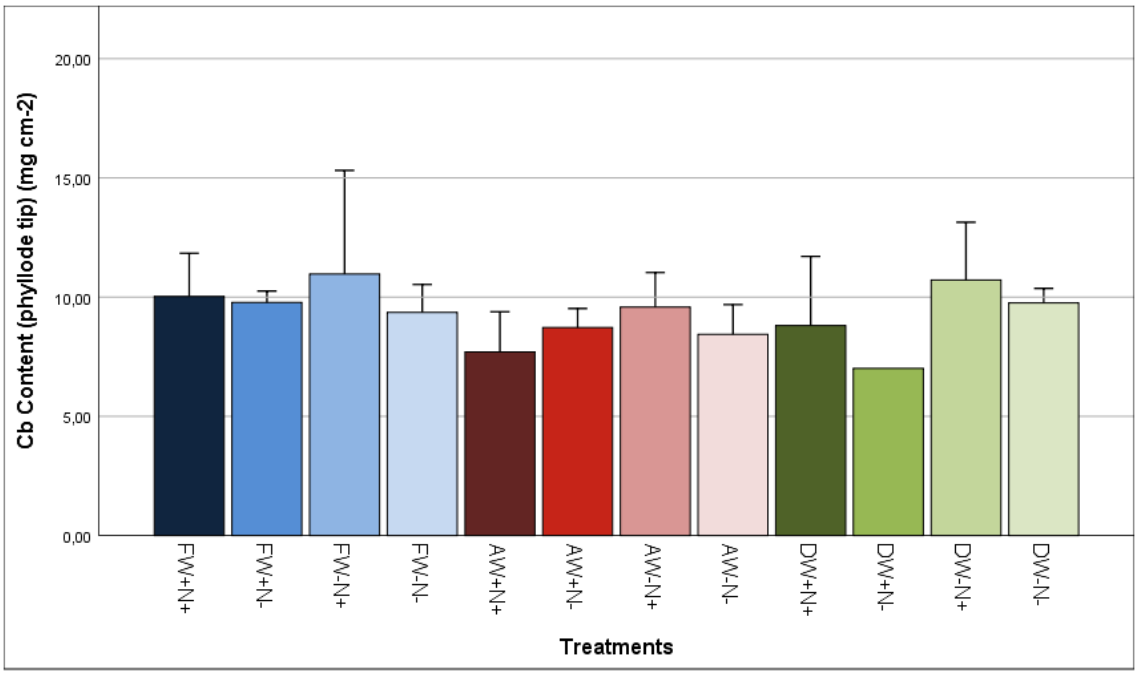


Figure 6.3: Average distribution of C_b content (phyllode tip) (mean ± 2SE) of *A. longifolia*, according to treatment combination (n= 55). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

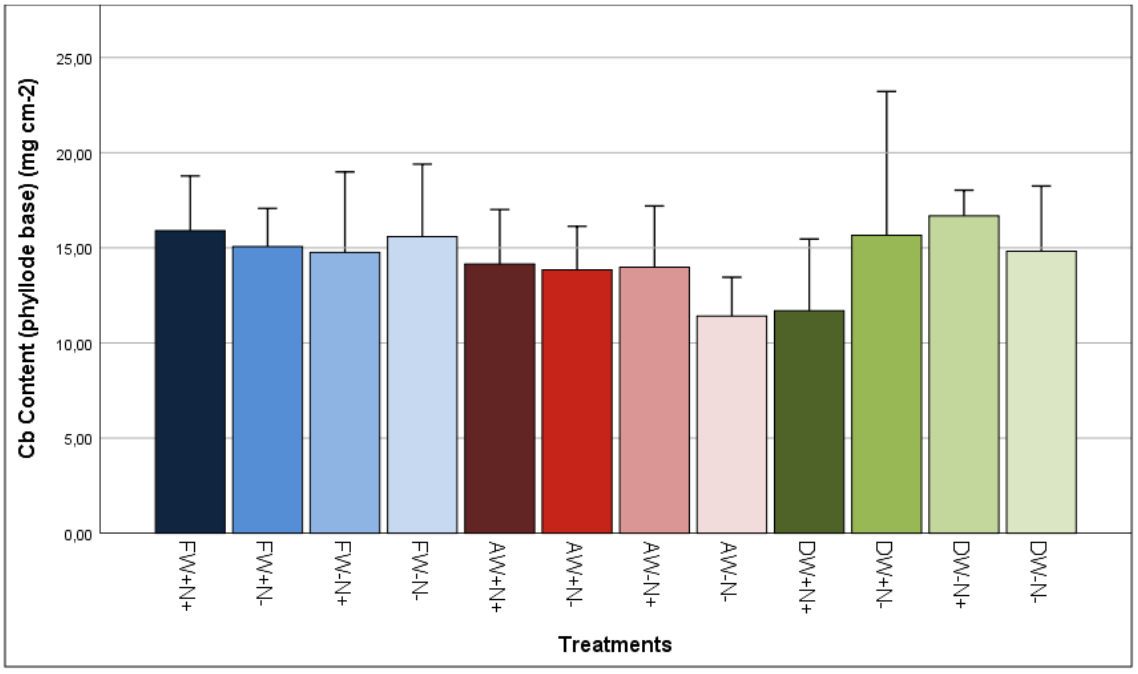


Figure 6.4: Average distribution of C_b content (phyllode base) (mean ± 2SE) of *A. longifolia*, according to treatment combination (n= 60). F, forest soil; A, agricultural soil; D, dune soil; W+N+, water at 70% field capacity/presence of nutrient solution; W+N-, water at 70% field capacity/absence of nutrient solution; W-N+, water at 30% field capacity/presence of nutrient solution; W-N-, water at 30% field capacity/absence of nutrient solution.

6.4 Appendix IV- Significance Analysis of Calibration Models

Table 6.5: F-test two samples for variance between SPAD-502 readings and absolute chlorophyll *a* (C_a) content at phyllode base.

	SPAD-502	C_a
Mean	54.189	38.886
Variance	52.813	60.847
Observations	59	59
gl	58	58
F	0.867	
P(F<=f) one-tail	0.295	
F critic one-tail	0.646	

Table 6.6: F-test two samples for variance between SPAD-502 readings and absolute chlorophyll *b* (C_b) content at phyllode base.

	SPAD-502	C_b
Mean	54.189	14.508
Variance	52.813	13.672
Observations	59	59
gl	58	58
F	3.862	
P(F<=f) one-tail	3.759E-07	
F critic one-tail	1.545	

Table 6.7: F-test two samples for variance between SPAD-502 readings and absolute total chlorophyll (C_{a+b}) content at phyllode base.

	SPAD-502	C_{a+b}
Mean	54.189	53.394
Variance	52.813	126.370
Observations	59	59
gl	58	58
F	0.417	
P(F<=f) one-tail	0.001	
F critic one-tail	0.646	

Table 6.8: F-test two samples for variance between SPAD-502 readings and absolute carotenoid content at phyllode base.

	SPAD-502	*Carotenoids*
Mean	54.189	8.476
Variance	52.813	3.187
Observations	59	59
gl	58	58
F	16.567	
P(F<=f) one-tail	2.471E-21	
F critic one-tail	1.545	

Table 6.9: F-test two samples for variance between SPAD-502 readings and absolute chlorophyll *a* (C_a) content at phyllode tip.

	SPAD-502	C_a
Mean	41.966	27.892
Variance	121.384	37.873
Observations	56	56
gl	55	55
F	3.205	
P(F<=f) one-tail	1.414E-05	
F critic one-tail	1.564	

Table 6.10: F-test two samples for variance between SPAD-502 readings and absolute chlorophyll *b* (C_b) content at phyllode tip.

	SPAD-502	C_b
Mean	41.966	9.391
Variance	121.384	6.007
Observations	56	56
gl	55	55
F	20.205	
P(F<=f) one-tail	1.873E-22	
F critic one-tail	1.564	

Table 6.11: F-test two samples for variance between SPAD-502 readings and absolute total chlorophyll (C_{a+b}) content at phyllode tip.

	SPAD-502	C_{a+b}
Mean	41.966	37.283
Variance	121.384	70.466
Observations	56	56
gl	55	55
F	1.722	
P(F<=f) one-tail	0.023	
F critic one-tail	1.564	

Table 6.12: F-test two samples for variance between SPAD-502 readings and absolute carotenoid content at phyllode tip.

	SPAD-502	*Carotenoids*
Mean	41.966	7.009
Variance	121.384	2.003
Observations	56	56
gl	55	55
F	60.578	
P(F<=f) one-tail	7.839E-35	
F critic one-tail	1.564	