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Effects of Arbuscular Mycorrhizal Fungi on the Vegetative Vigor of *Ailanthus altissima* (Mill.) Swingle Seedlings under Sustained Pot Limitation

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Abstract: In order to invade new ecosystems, invasive alien plants need to cope with different microbial communities. Whilst the ability to avoid antagonists is well recognized, the opportunity to establish mutualistic associations is less known, even in widespread invasive species such as *Ailanthus altissima* (Mill.) Swingle. We sought to evaluate whether the beneficial effects of arbuscular mycorrhizal fungi (AMF) on *Ailanthus* seedlings are maintained over time, under prolonged pot limitation. We compared three-month-, three-year- and four-year-old mycorrhizal seedlings grown in natural forest soil (NT) with seedlings grown in sterilized (ST) and non-mycorrhizal (NM) soils, in pots of 3.4 L (22 × 15 cm). Growth parameters and leaf traits were assessed, including carbon (δ^{13}C) and nitrogen (δ^{15}N) stable isotope compositions. NT seedlings showed relatively higher vigor in the early stage but, subsequently, the benefits provided by AMF were lost. Interestingly, mycorrhizal seedlings consistently showed about 2‰ δ^{13}C enrichment, relatively to the other treatments. Negative linear relationships between leaf δ^{13}C and N content were found. Higher photosynthesis rates and WUE are the likely causes of the early enhanced growth in mycorrhizal seedlings. The symbiotic relationship between AMF and *Ailanthus* could be driven by resource availability. Greater insights into such aspects could provide an improved perspective on the ecological limits of *Ailanthus*.

Keywords: carbon and nitrogen stable isotopes; invasive species; Mediterranean forests; mycorrhizae; soil microbiota; Tree of Heaven

1. Introduction

The invasive nature displayed by certain alien plant species is generally attributed to differing physiological, ecological, and anthropic reasons, and is rarely dependent upon a single factor [1,2]. In order for invasive alien species to establish in new ecosystems, they would need to effectively interact with a different local biota [3]. In recent years, increasing attention has been paid to biotic interactions between the introduced species and the recipient ecosystem [4], including plant-soil microbe relationships, which strongly affect plant performance and competitive ability [5,6]. One general assumption is that invasive plant species may establish, on the whole, more positive interactions with soil biota than native co-occurring species. For instance, exotic species may experience either partial or complete release of specialist enemies in co-evolution in the native range [7,8]. Alteration of the direction and amplitude of feedbacks with soil microorganisms has been found to disrupt the

density-dependent mechanisms which regulate natural populations of woody species. One of the reasons which supposedly underlies the greater stand density found in invaded areas, compared to the native range of *Acer* spp. [9], is a strong positive relationship with soil biota in the secondary range. Furthermore, substantial changes in the structure, functioning, and species assemblage of belowground soil communities are usually associated with plant invasions [10,11].

The widespread occurrence in extremely different pedological and microbial conditions, and the ability to reach considerable densities in invaded areas, make *Ailanthus altissima* (Mill.) Swingle one of the best candidates in the investigation of alien plant–soil-microbe interactions. It is a fast-growing, pioneer alien plant species native to China and North Vietnam, which was first introduced as an ornamental in Europe in the middle of the 18th century [12]. *Ailanthus altissima* is one of the most invasive tree species in the world, especially in temperate and Mediterranean-type ecosystems, where it has invaded disturbed and synanthropic habitats, as well as semi-natural and natural woodlands, shrublands, river beds, and grasslands [13]. Its broad ecological plasticity enables it to tolerate a wide range of climatic, soil, and sub-optimal ecological conditions [14,15]. In forest habitats, *A. altissima* takes particular advantage of natural or anthropic disturbances and gap development [16]. A number of biological and ecophysiological reasons have been taken into account when attempting to explain its invasive nature and competitive ability over native coexisting species. A lack of natural enemies, very rapid initial growth, effective vegetative spread by means of lateral shoots, and high resilience to human disturbance (fire, grazing, etc.) are considered as particularly relevant [17,18].

Only recently, has attention also turned to soil microbial communities and belowground biotic interactions as possibly playing a very important role in explaining not only the dominance of *Ailanthus* at a small scale, but also its resistance to abiotic stress and its notable ability to acquire limited resources in Mediterranean environments. The very high tree densities obtained by *A. altissima* may also depend upon allelopathic effects exerted on many broadleaves and evergreen species, with a strong species-specific effect [19,20]. The ability to elude antagonistic organisms (pathogens, pests, etc.) in the soil, whereas at the same time benefitting from mutualist organisms (mycorrhizal fungi, growth promoting bacteria, etc.), seems to be one of the winning strategies adopted by some invasive tree species, such as *Robinia pseudoacacia* L. [21]. Despite the current lack of sufficient knowledge in this regard, *Ailanthus* could be able to employ a similar ecological strategy.

Due to their low specificity with host plants and widespread occurrence among vascular plants [22], arbuscular mycorrhizal fungi (AMF) were considered, until recently, relatively less influential than other biological aspects in triggering alien plant invasiveness [5]. Accordingly, their effective importance for most woody alien species is still largely unknown [21,23]. However, recent evidence has shown that AMF could be more important than previously thought. AMF are common root symbionts that can increase host-plant establishment and growth in stressful environments, such as marginal, nutrient-poor, and anthropogenic soils [24]. A possible role of endomycorrhizae in enhancing the competitive ability of *A. altissima* stems from recent observations showing the facultative nature of this plant and its ability to establish mutualistic symbiosis with AMF in different habitats, both in North American and European secondary ranges [25,26]. Such a capacity could be determinative as regards the uptake of necessary resources, enhancing stress tolerance as well as improving the fitness and growth of seedlings in the field: all traits which confer high competitiveness. The existence of a presumably habitat-based control of the type of mycorrhizal colonization may allow *A. altissima* to regulate exchange rates with symbiotic fungi, thereby ensuring that any parasitic relationship is, seemingly, avoided. Such a regulatory control over the host species would confer a certain plasticity in colonizing environments with differing limiting factors for *A. altissima* establishment.

Recent research has shown that *A. altissima* mycorrhizal seedlings, growing in natural Mediterranean forest soil, grow faster and accumulate much more biomass than non-mycorrhizal seedlings growing in sterilized soil and in soil lacking only mycorrhizal propagules [26]. Mycorrhizal dependency was calculated up to 84%, suggesting that AMF may play a crucial role in promoting *A. altissima* seedling establishment, growth performance, and competitive interactions

within Mediterranean forest ecosystems. Therefore, any variation in the mutualistic pattern developed by *A. altissima* we would expect to be reflected in different resource-use strategies. In this respect, the analysis of stable isotope fractionations of elements such as C or N, may provide information on plant ability to regulate carbon, water and nitrogen economy. Stable carbon isotopes are increasingly used to assess physiological state and water-use efficiency (WUE) in C_3 plants [27,28], including invasive ones [29].

Under progressively limiting pot conditions in a greenhouse experiment, we aimed at evaluating, by means of isotopic analyses on leaf dry matter over different years, whether long-term growth and WUE of *A. altissima* seedlings were affected by soil biotic conditions. Natural soil (not treated, including the whole soil biota), sterilized soil (abiotic soil), and soil sterilized and enriched with a filtered soil extract (including soil biota without mycorrhizal propagules) were compared. Short-term information provided through stable isotope applications is generally retained in fast turnover metabolites. For instance, leaf soluble sugars reflect the temporary short conditions during which they are synthesized [30,31]. The whole crown leaf dry matter retains a temporal extended, assimilation-weighed information. Especially in tree species, the position and ageing of the leaf within the crown can determine very diverse assimilation rates. This information is mostly recorded in the structural leaf carbon, although a confounding effect of fast turnover metabolites and of compounds other than carbohydrates may be relevant in some conditions. In the present experiment, leaf sampling at the end of the vegetative cycle makes such an eventuality unlikely. Furthermore, the long-term significance of our approach is corroborated by leaf samplings repeated over several years. This allowed us to describe the stable isotope physiology over the long-term period; i.e., looking at the physiological responses over succeeding growing seasons, under the increasingly limiting conditions due to confined pot volume. In particular, by comparing three-month-old seedlings with three-year- and four-year-old seedlings, we aimed at verifying whether differences in growth and leaf parameters, as well as isotopic signatures, are maintained between treatments and over time.

2. Materials and Methods

2.1. Nursery Propagation of Seedlings and Experimental Design

The experimental trial was established in August 2012 at the experimental fields belonging to the Department of Agricultural, Food, and Forest Sciences at the University of Palermo. Plant material was propagated in pots in a greenhouse during a previous experiment addressing the influence of mycorrhizal fungi on *Ailanthus* seedling performance. More details on soil collection, preparation methods of soil treatments, plant propagating material, and growing conditions are given in Badalamenti et al. [26]. In brief, seeds were sown in 3.4 L pots (22 × 15 cm), filled with a limestone soil containing 1.2% organic carbon and 0.8‰ nitrogen. The experiment was carried out in greenhouse and seedlings were grown in three different soil conditions: natural soil (natural, not treated: NT), which contained the whole soil microbiota (including naturally occurring mycorrhizal fungi), sterilized soil (sterilized: ST), and sterilized soil enriched with a filtered soil extract (including the whole soil biota but without mycorrhizal propagules) (non-mycorrhizal soil: NM). Examination of fresh roots after three months confirmed substantial colonization by AMF in roots from NT pots, while no evidence of endomycorrhizal structures was found in either ST or NM pots. During the course of the experiment, all pots were irrigated twice a week, without fertilization. In order to evaluate seedling physiological state, biometric, foliar sampling surveys, and stable isotope analyses were carried out in three-month-old, three-year-, and four-year-old seedlings. A total of 20 seedlings per treatment (NT, NM, and ST) were considered: 10 replicates per treatment for three-month-old seedlings and 5 replicates each for the three-year- and four-year-old seedlings.

2.2. Biometric Parameters

Leaf collection was carried out at the end of the growing season (early November) in 2012, 2015, and 2016. We considered a time span long enough to evaluate whether the beneficial effects attributable to mycorrhizal colonization were maintained over time, as three-month-old, three-year-, and four-year-old seedlings were compared. As regards the whole plant, height and stem diameter were considered. Leaf traits included: leaf number (N), leaf area (LA), leaf dry weight (LDW), leaf mass area (LMA), and specific leaf area (SLA). LA was measured by using a leaf area meter (Delta T Devices, USA), and LDW after stable weighing at 80 °C. LMA and SLA were calculated based on the ratio LDW over LA and vice versa, respectively. Total N content was measured by means of an elemental analyzer (NA 1500, Carlo Erba, Italy).

The relative growth rate of leaf biomass (RGR) was calculated as [32,33]

$$\ln W_2 - \ln W_1 / (t_2 - t_1), \tag{1}$$

where W_2 and W_1 are the final and the initial leaf biomass (in g), respectively, and $t_2 - t_1$ is the time interval (days). The initial biomass value was considered to be the mean seed weight, coming from data from Southern France [15], and corresponding to 0.011 g for all treatments.

2.3. Stable Isotopes Analysis

Stable isotope ratios were determined using a continuous-flow, triple-collector, isotope ratio mass spectrometer (ISOPRIME, GV, Manchester, UK). Finely powdered sub-samples of dried leaves ($\approx$0.5 mg for C; $\approx$2.5 mg for N) were quantitatively combusted in an elemental analyzer (Model NA 1500, Carlo Erba, Milan, Italy). Nitrogen oxides produced in the oxidation reactor were reduced to elemental N in a reactor filled with elemental copper and operated at 650 °C. Both CO_2 and N_2 were transferred in helium flow to the mass spectrometer for the determination of the isotopic ratios (R), $^{13}C/^{12}C$ and $^{15}N/^{14}N$, respectively. The isotopic compositions of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) of the samples were determined, according to the definitions described in Farquhar et al. [34], as δ notation

$$\delta = (R_s - R_{std}) / R_{std}, \tag{2}$$

where R_s is the isotope ratio of the sample and R_{std} is the isotope ratio of the international standard. Isotope compositions were anchored to IAEA international standards on the VPDB and atmospheric nitrogen scales for $\delta^{13}C$ and $\delta^{15}N$ determination, respectively. The precision of measurement, expressed as standard deviation when measuring a sample 10 times, was 0.1‰ for $\delta^{13}C$ and 0.2‰ for $\delta^{15}N$.

2.4. Statistical Analysis

A one-way ANOVA was carried out to determine the effect of soil treatment (N, ST, and NM) on plant growth variables, leaf traits and isotopic composition. Significance was determined at the 95% level of confidence. Before performing ANOVA analysis, normality and homogeneity of variance of data were verified. Our samples, split by treatment and year, were normally distributed, using the non-parametric skewness (mean-median)/stdev, for all possible combinations. All the conditions were met, including heteroscedasticity (R output). We also performed two-way ANOVA tests on $\delta^{13}C$ and N% distributions. Significant interaction of treatment and year on both variables was found ($p = 0.020$ for $\delta^{13}C$ and $p < 0.001$ for N%). The year variable was treated as a factor in a linear model (the R lm function): $\delta^{13}C$ as a linear combination of N%, TYPE and YEAR. An ordinary linear regression analysis ($\delta^{13}C$ vs. N×LMA and $\delta^{13}C$ vs. %N) was performed to explore possible relationships between measured variables: on each treatment for the different years, on pooled treatments for each year and on pooled treatments for pooled years. Statistical analysis was performed using Systat Software, Inc. 2009 (version no. 13.00.05, San Jose, CA, USA).

3. Results

3.1. Growth Parameters

Differences were found in seedling height and stem diameter between treatments in 2012, parameters being significantly higher in natural soil, and significantly lower in non-mycorrhizal soil (Table 1). Such a trend was not maintained over time regarding seedling height, which did not differ in the following years. Stem diameter was still significantly higher in natural soil compared to the other two treatments in 2015, whereas it was significantly lower only in non-mycorrhizal soil in 2016.

Table 1. Growth parameters of *A. altissima* seedlings. Means followed by different letters in superscript within each year are significantly different at $p < 0.05$ after Tukey's HSD test.

Soil Treatment	Height (cm)			Stem Diameter (cm)		
Year	2012	2015	2016	2012	2015	2016
Natural (NT)	11.0 [a]	57.2 [a]	75.1 [a]	0.6 [a]	0.9 [a]	1.0 [a]
Sterilized (ST)	9.9 [b]	61.2 [a]	76.3 [a]	0.4 [b]	0.6 [b]	0.9 [a]
Non Mycorrhizal (NM)	7.7 [c]	59.0 [a]	76.2 [a]	0.3 [c]	0.6 [b]	0.8 [b]

3.2. Leaf Traits

Significant differences in leaf number and leaf area were found between treatments only in 2012. Leaf number was significantly lower in NM treatment. Leaf area was significantly higher in NT treatment. Significant differences in physiological leaf traits between treatments were detected only in 2015, when LMA was significantly higher in NT treatment, whereas ST and NM treatments were not statistically different (Table 2). The opposite situation holds for SLA, this parameter being significantly lower in NT soil only in 2015. *A. altissima* seedlings developed thicker leaves in 2015 and 2016 compared to three-month-old seedlings.

In 2012, the relative growth rate of leaf biomass was significantly higher in NT treatment and significantly lower in NM treatment (Table 3). In 2015, the trend was inverted, RGR being significantly higher in NM treatment and significantly lower in NT treatment. ST treatment did not differ from NT and NM treatments. In 2016, no significant difference was found in any investigated parameter.

3.3. Stable Isotope Analysis

Isotopic analyses displayed significant differences between treatments (Table 4). Seedlings grown in NT soils constantly had significantly higher $\delta^{13}C$ over the three investigated years, compared to the other soil treatments. Irrespective of the year, there were no significant differences in $\delta^{13}C$ when comparing plants grown in ST and NM soils. In contrast, leaf $\delta^{15}N$ values did not show any clear pattern, with significantly lower values in NM soil in 2015 only. Nitrogen content (%) was significantly higher in NT soil in 2012 only.

Within each year, regression analysis showed weak relationships between $\delta^{13}C$ and leaf N content, regardless of the treatment. However, when pooling data by all years in each treatment, highly significant regressions were found when analyzing responses in $\delta^{13}C$ vs. leaf N content. Accordingly, a strong negative relationship was found when grouping data from treatments and years all together (Table 5; Figure 1). Weaker relationships were found between $\delta^{13}C$ vs. N×LMA. We obtained a value of r which was between -0.96 and -0.84 within homogenous treatment classes, and $r = -0.81$ when combining all three classes.

Table 2. Leaf traits. Means followed by different letters in superscript within each year are significantly different at $p < 0.05$ after Tukey's HSD test.

Soil Treatment	Leaf Number (N)			Leaf Area (cm^2)			Leaf Mass Area (g m^{-2})			Specific Leaf Area (cm^2 g^{-1})		
Year	2012	2015	2016	2012	2015	2016	2012	2015	2016	2012	2015	2016
Natural (NT)	9.9 [a] ± 0.6	10.2 [a] ± 1.1	10.4 [a] ± 0.5	160.82 [a]	1539.90 [a]	1607.01 [a]	22.5 [a]	51.8 [a]	32.0 [a]	448.7 [a]	199.4 [b]	326.1 [a]
Sterilized (ST)	8.4 [a] ± 0.3	9.8 [a] ± 0.6	10.2 [a] ± 0.7	87.35 [b]	1389.73 [a]	1516.84 [a]	22.4 [a]	35.8 [b]	29.2 [a]	456.0 [a]	284.5 [a]	350.4 [a]
Non Mycorrhizal (NM)	7.3 [b] ± 0.2	8.6 [a] ± 1.0	9.2 [a] ± 1.2	48.39 [c]	1325.63 [a]	1480.11 [a]	24.1 [a]	41.4 [b]	25.5 [a]	419.8 [a]	261.6 [a]	404.4 [a]

Table 3. Relative growth rate of leaf biomass. Means followed by different letters in superscript within each year are significantly different at $p < 0.05$ after Tukey's HSD test.
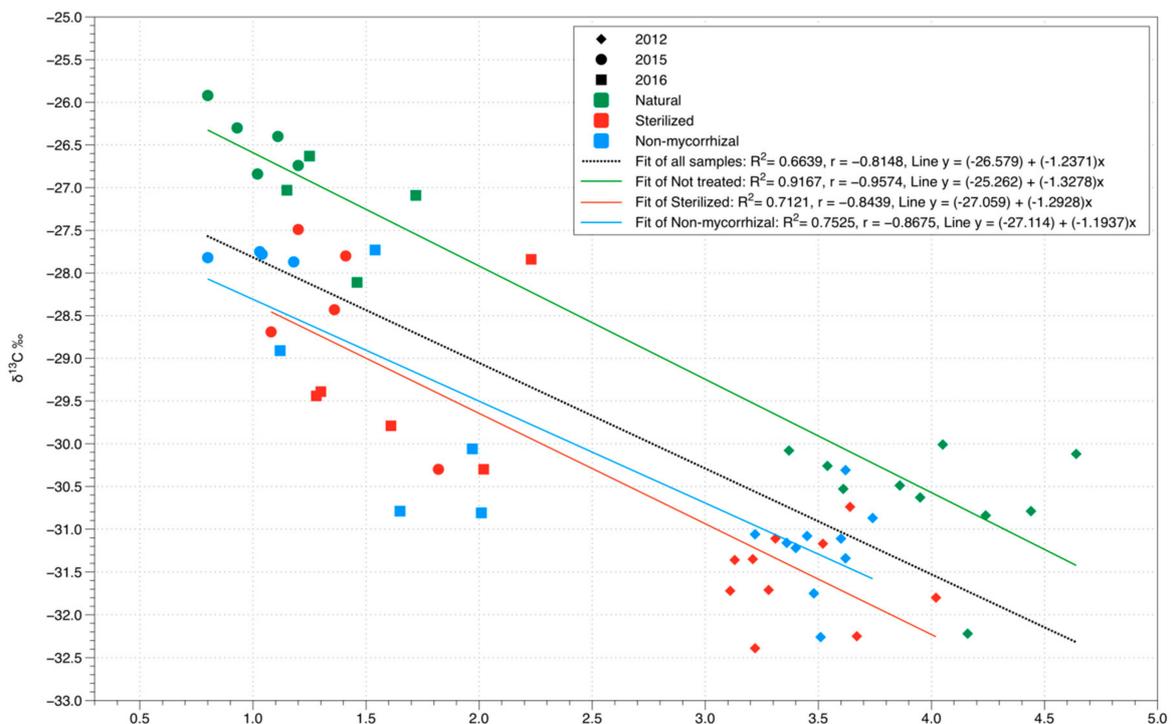
Soil Treatment	RGR (Days^{-1})		
Year	2012	2015	2016
Natural (NT)	0.0347 [a] ± 0.0007	0.0026 [b] ± 0.0003	0.0004 [a] ± 0.0002
Sterilized (ST)	0.0279 [b] ± 0.0016	0.0030 [ba] ± 0.0001	0.0005 [a] ± 0.0001
Non Mycorrhizal (NM)	0.0233 [c] ± 0.0024	0.0035 [a] ± 0.0006	0.0004 [a] ± 0.0001

Table 4. Carbon and nitrogen isotope compositions, nitrogen percentage, and nitrogen content on a leaf area basis, in leaves of *A. altissima* seedlings. Means followed by different letters in superscript within each year are significantly different at $p < 0.05$ (Tukey's HSD test).

Soil Treatment	δ^{13}C (‰)			δ^{15}N (‰)			N (%)			NxLMA (g m^{-2})		
Year	2012	2015	2016	2012	2015	2016	2012	2015	2016	2012	2015	2016
Natural (NT)	−30.6 [a]	−26.4 [a]	−27.2 [a]	8.2 [a]	9.2 [a]	7.7 [a]	4.0 [a]	1.0 [a]	1.4 [a]	0.9 [a]	0.5 [a]	0.4 [a]
Sterilized (ST)	−31.6 [b]	−28.5 [b]	−29.4 [b]	7.9 [a]	9.5 [a]	8.1 [a]	3.4 [b]	1.4 [a]	1.7 [a]	0.8 [b]	0.5 [a]	0.5 [a]
Non Mycorrhizal (NM)	−31.2 [b]	−27.8 [b]	−29.7 [b]	7.7 [a]	7.7 [b]	8.6 [a]	3.5 [b]	1.0 [a]	1.7 [a]	0.8 [ab]	0.4 [a]	0.4 [a]

Table 5. Coefficients of determination (r^2) for linear regressions of δ^{13}C vs. N×LMA and δ^{13}C vs. %N, in each treatment in the different years, in the pooled treatments by year and in the pooled treatments by pooled years.

Treatment	Year	δ^{13}C vs. N×LMA	δ^{13}C vs. %N
Natural	2012	0.14	0.06
Sterilized	2012	0.20	0.00
Non-mycorrhizal	2012	0.28	0.03
Natural	2015	0.16	0.60
Sterilized	2015	0.09	0.57
Non-mycorrhizal	2015	0.45	0.00
Natural	2016	0.42	0.10
Sterilized	2016	0.16	0.14
Non-mycorrhizal	2016	0.02	0.35
All treatments	2012	0.33	0.11
All treatments	2015	0.05	0.57
All treatments	2016	0.03	0.14
All treat. all years	2012–2015–2016	0.34	0.67

Figure 1. Linear regressions showing the relationship between δ^{13}C (‰) and N content (%) for all treatments and years.

4. Discussion

Symbiotic mutualistic relationships in the study of invasive process of new ecosystems by alien plant species is gathering increasing attention [35,36]. While avoiding antagonists is a trait commonly recognized in most invasive plants, the simultaneous ability to acquire mutualistic organisms is less generalized and, overall, less known [21]. Mycorrhizal potted seedlings of *Ailanthus altissima*, grown in natural Mediterranean forest soil, showed higher growth rates than seedlings grown in sterilized or in non-mycorrhizal soils [26]. However, observations were strictly confined to very juvenile stages, with no information on subsequent dynamics of acclimation to different soil microbiological

conditions. Here we provide physiological information on plant responses at the age of three months, three, and four years, considering the effects of increasing limitation due to a fixed pot size of 3.4 L.

Firstly, we found that the faster development of mycorrhizal seedlings was gradually reduced in time, especially in terms of plant height. Three- and four-year-old plants did not differ anymore in height, whilst stem diameter was significantly higher in NT soil up to three-year-old seedlings. ST treatment parameters were found to be constantly higher than NM treatment; this may be at least partially due to initial increased nutrient availability resulting from the soil sterilization process [37]. Additionally, the possible presence of pathogens could have caused some negative effects in NM treatment. Some degree of structural leaf diversity was evident only after three years of growth, with significantly thinner leaves in both ST and NM treatments, as indicated by LMA or SLA values. In all treatments, leaves became relatively thicker and smaller with ageing; these traits are common in plants that face increasing environmental limitations [38,39].

Pot restriction caused an increase in carbon isotope composition of leaf dry matter in three-year- and four-year-old seedlings, as compared to three-month-old seedlings. Plants growing in NT soils showed more than 4‰ enrichment in $\delta^{13}C$, compared to approx. 3‰ enrichment in the other two treatments. This indicates large WUE variation over time and between treatments. Analysis of carbon isotope discrimination in C_3 plants is a well-known proxy of plant WUE [27,30,34]. Given the constancy of $\delta^{13}C$ of atmospheric CO_2, any driver leading to variations in the ratio of intercellular to atmospheric CO_2 concentrations (C_i/C_a) would cause an effect on $\delta^{13}C$ of photoassimilates, following an inverse linear relationship. Thus, a decrease in C_i owing to reduced stomatal conductance or increased photosynthetic capacity would result in ^{13}C enrichment (less negative $\delta^{13}C$) of photosynthetic products (related in both circumstances to an increase in WUE [27,31]). The concomitant decrease in leaf N content with ageing, both on a dry matter and leaf area basis, strongly indicates that enriched $\delta^{13}C$ values are mostly explained by stomatal closure rather than by increased photosynthetic capacity [40–43]. Furthermore, mycorrhizal seedlings had significantly higher $\delta^{13}C$ values in leaf dry matter than seedlings growing in ST or NM soil. Importantly, $\delta^{13}C$ was the only leaf parameter analyzed throughout this study showing a constant pattern over all the period. Such a finding seems to attribute a significant role to endomycorrhizae in that they are related to the expression of greater WUE ($\delta^{13}C$ enrichment in leaf dry matter). The other two soil microbial conditions, i.e., ST and NM treatments, did not show comparative differences in foliar $\delta^{13}C$. Other researchers have shown similar effects of arbuscular mycorrhizal fungi [44,45]. However, knowledge is still limited and no generalized trend has been observed; in other woody species, either no effect [46] or even a reverse effect [45] have been described. In our experiment, any advantage gained by higher aboveground growth rates at seedling stage in AMF infected plants (NT treatment) was offset by reduced stomatal conductance.

Three-month-old seedlings growing in NT soil had significantly higher values of both $\delta^{13}C$ and leaf N content when compared to ST and NM treatments. In the absence of soil volume limitation, such observation would suggest that differences in photosynthetic capacity of *A. altissima* seedlings are primarily driven by its root microbiota. Higher photosynthesis rates (see higher growth rates in Table 1) and intrinsic WUE (enriched values of $\delta^{13}C$ of dry matter) were the likely reasons for enhanced growth ability in the mycorrhizal plants, thus providing evidence of clear beneficial effects mediated by soil fungi to an invasive plant species. This mutualistic association is effective in enhancing the nutritional status of NT seedlings, especially during the first year (see N content in Table 3).

The negative relationship linking leaf N content and $\delta^{13}C$ (Figure 1) is unusual in that it is the reverse of what is commonly expected. It is recognized that soil N availability may change carbon isotopes ratios due to its influence on gas exchange parameters and photosynthesis, thus affecting the relationship between $\delta^{13}C$ and WUE of C_3 plants [47]. In particular, higher nitrogen content may decrease the ratio C_i/C_a, by enhancing photosynthetic capacity or carboxylation efficiency [27], or by reducing stomatal conductance or by both processes [32,48,49]. In any case, the positive relationship between N content and WUE has been widely reported in many plant species [49,50], including woody species [48,51] and invasive woody plants [52]. However, nitrogen level may considerably

shift such a pattern in that a strong positive effect on WUE could be observed only under high N supply, which caused a stronger influence on stomatal conductance [53]. Interestingly, we found a total reversal of the relationship between N content and $\delta^{13}C$ over time, from strong positive in three-month-old seedlings and high leaf N, to strong negative with low leaf N. Such data indicate enrichment in ^{13}C at relatively lower N contents during seedling ageing. More commonly, a decrease in leaf N content is associated with a depletion of ^{13}C in the photosynthates of C_3 plants.

In the present experiment, plants had to cope with increased limitations due to root constriction in a limited soil volume, in the absence of any fertilization [54]. This caused a general rearrangement of the photosynthetic apparatus, irrespective of the soil treatment, as demonstrated by the remarkable parallelism of the regression lines in Figure 1 [40,42]. The decrease in leaf nitrogen content seemed to drive or accompany this photosynthetic rearrangement, and is unquestionably linked to increased environmental limitations faced by seedlings. Other than nutritional aspects, such limitations likely concern plant water status. A general increase in intrinsic WUE was indicated by $\delta^{13}C$ values which were found to be less negative, combined with a reduction in leaf N content. It is assumed that more than half of leaf nitrogen is involved in photosynthetic proteins and components, Rubisco alone accounting for 20–30% [55–57]. Decreasing photosynthetic capacity would imply decreasing intrinsic WUE due to an increased C_i/C_a ratio. As photosynthetic capacity and leaf nitrogen content are strictly linked [43,58], increased WUE linked to decreased nitrogen content must be related to stomatal effects. In our experiment, the increased stomatal closure likely overcame the counterbalancing metabolic effects due to nitrogen variation.

In 2012, RGR analysis revealed significantly higher RGRs of leaf biomass in natural soil grown seedlings compared to sterilized or non-mycorrhizal theses (Table 3). Conversely, in 2015, RGR largely decreased in all treatments; especially in NT treatment, which showed significantly lower values. It seems the initial advantage provided by the mycorrhizal relationship in the natural soil treatment is progressively lost due to pot-based limitation in resource availability. This suggests a general decrease in primary productivity, likely driven by both stomatal and biochemical limitations, as postulated on the basis of carbon stable isotopes analysis.

We did not find any clear relationship between leaf N content, which differed between treatments only at the three-month-old seedling stage, and its isotopic composition ($\delta^{15}N$), which was different only at the three-year-old stage. Such evidence seems to further confirm the current difficulty in attributing a clear role to intraspecific variation in leaf $\delta^{15}N$ between endomycorrhizal and non-mycorrhizal plants [59]. Endomycorrhizae might be responsible for the transfer of ^{15}N-depleted organic matter from AMF to host plant. Such a pattern has been more widely and constantly observed in ectomycorrhizal symbionts, which are capable of reducing ^{15}N values up to −12‰ [60,61], and able to account for approx. 25% of total variation in ^{15}N leaf content [44,52]. However, endomycorrhizae seem to exert a weaker and less predictable effect than widely attributed to ectomycorrhizae [61,62]. Handley et al. [44] found that fungal endosymbionts are responsible for the transfer of ^{15}N-enriched organic matter with an increase in ^{15}N leaf content of as much as 3.5‰. Another field study [63] ascertained large variations in the effect of endomycorrhizae based on habitat type, ranging from depleted (−4.1‰) to enriched (+2.2‰) ^{15}N foliar values. Such results imply that a generalized effect of AMF on leaf nitrogen isotopic signature does not exist; rather this relationship is strongly influenced by climatic and other local factors. In a recent review, Hodge and Storer [64] highlighted the widespread occurrence of AMF in nature and their role in establishing symbiotic interactions with a multitude of plant species. Although the AMF function in facilitating nitrogen uptake in plants is clear, their N contribution to plants is known to be highly variable [65]. The reasons for this variability are still unclear.

Observations performed in this research refer to small and confined systems, namely pot-grown plants hosting different soil microbial communities. Abrupt temporal changes in resource availability might easily occur in such restricted conditions. Said otherwise, the mutualistic symbiosis clearly apparent in three-month-old seedlings [26] may have shifted in subsequent years towards different

forms of neutral or even antagonistic relationships [66]. It is known that below some threshold value, nutrient limitation may foster competition between host plant and fungal symbiont, significantly affecting the direction and magnitude of symbiotic relationships [67]. Due to unbalanced plant source/sink relationships and to likely water and nutrient limitations in the pot environment, a competitive network of interspecific relationships might have occurred, obscuring the initial variability observed in terms of growth, intrinsic WUE, and leaf N content in the treatments.

5. Conclusions

The observation of long-term acclimation of *A. altissima* potted plants revealed that improved physiological status of mycorrhizal seedlings is strongly affected by pot limitation. Both growth performances and, most likely, fitness of *A. altissima* seedlings are dependent upon intrinsic site characteristics. Pot-based limitation in resource availability could have overwhelmed any initial advantage gained in plant growth and vigor (owing to the effectiveness of AMF). Although the present experiment refers only to controlled environmental conditions, the evidence collected may stimulate new experimental hypotheses to be tested in the near future along gradients of field conditions. In particular, our results point to a certain vulnerability of *A. altissima* in supporting positive mutualisms with AMF species should resource availability become inadequate for the abundant requirements of this pioneer and fast growing invasive species. Greater insights into these aspects could provide an improved perspective on the ecological control and management of *A. altissima* invasion.

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